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To shift or not to shift: Low density lipoprotein receptor-related protein 1 and the plasminogen activators gate phase shifting in the mammalian circadian clock

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To the Graduate Council:

I am submitting herewith a dissertation written by Joanna Marie Cooper entitled "To shift or not to shift: Low density lipoprotein receptor-related protein 1 and the plasminogen activators gate phase shifting in the mammalian circadian clock." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry and Cellular and Molecular Biology.

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To shift or not to shift: Low density lipoprotein receptor-related protein 1 and the plasminogen activators gate phase shifting in the mammalian circadian clock

A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Joanna Marie Cooper
May 2018
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ABSTRACT

Here, we present data supporting low density lipoprotein receptor-related protein-1 (LRP-1) and urokinase plasminogen activator (uPA) involvement in the suprachiasmatic nucleus (SCN), the primary mammalian circadian pacemaker. Previous work using extracellular recordings of SCN neurons in ex vivo hypothalamic slices demonstrated that tissue-type plasminogen activator (tPA) gates glutamate-induced phase shifts via plasmin-dependent maturation of brain derived neurotrophic factor (BDNF) and subsequent tropomyosin receptor kinase B (TrkB) receptor activation. Here, we find first, that tPA knockout mice (tPA−/−; B6.129S2-Plattm1Mlg/J) exhibit minimal phase shifting deficits in vivo and in vitro, and that uPA compensates for the lack of tPA to enable phase shifts in these mice. Intriguingly, the data support tPA, but not uPA, acting via BDNF maturation, suggesting functional compensation achieved through differential mechanisms. Second, we find that LRP-1 also regulates SCN phase shifting. Inhibiting LRP-1 with receptor associated protein (RAP) or anti-LRP-1 antibody prevents glutamate-induced phase delays and advances in neuronal activity rhythms in vitro at ZT16 and ZT23, respectively. We then turned our attention to potential interactions between tPA and LRP-1, and through three lines of evidence demonstrate that tPA proteolytic activity is not necessary for LRP-1’s permissive effect on phase shifting: 1) RAP inhibits phase shifts in tPA+/− SCN, 2) inhibiting LRP-1 does not impact BDNF maturation, or 3) Trk receptor phosphorylation on Y680/681. Surprisingly, inhibiting LRP-1 with RAP changes N-Methyl_D-aspartic acid receptor (NMDAR) phosphorylation patterns in the SCN in vitro, by decreasing phosphorylation on S1480 of NR2B subunits. Finally, we evaluated uPA and tPA expression and proteolytic activity across the circadian day, and LRP-1 expression and phosphorylation patterns. We find evidence of circadian rhythms in tPA expression but not proteolytic activity, no rhythms in uPA expression or proteolytic activity, and potential diurnal variations in αLRP-1 but not βLRP-1 subunits. Additionally, uPA activity and βLRP-1 expression exhibit changes that correlate with the time slices are maintained in vitro, suggesting that a response to slicing injury may occlude an accurate view of expression patterns in the SCN in vitro. Collectively, the data presented here implicate uPA and LRP-1 in the processes gating glutamate-induced phase shifts in the SCN.
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LIST OF ABBREVIATIONS

hour (h)
suprachiasmatic nucleus (SCN)
gamma-Aminobutyric acid (GABA)
gastrin-releasing peptide (GRP)
vasoactive intestinal peptide (VIP)
vasopressin (AVP)
2-deoxyglucose (2-DG)
transcriptional-translational-posttranslational negative feedback loop (TTFL)
Period (Per)
Cryptochrome (Cry)
pituitary adenylate cyclase-activating peptide (PACAP)
α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA)
N-Methyl-D-aspartic acid receptors (NMDA)
Ca²⁺/calmodulin-dependent protein kinase II (CaMKII)
Phosphorylated CaMKII (pCaMKII)
neuronal nitric oxide synthase (nNOS)
nitric oxide (NO)
ryanodine receptors (RyRs)
guanylate cyclase (GC)
cyclic guanosine monophosphate (cGMP)
cAMP response element binding protein (CREB)
cAMP response element (CRE)
mitogen-activated protein kinase (MAPK)
protein kinase A (PKA)
Src family kinase (SFK)
neural cell adhesion molecule (NCAM)
L1 cell adhesion molecule (L1-CAM)
extracellular matrix (ECM)
long term potentiation (LTP)
long term depression (LTD)
Brain-derived neurotrophic factor (BDNF)
mature BDNF (mBDNF)
uncleaved BDNF (proBDNF)
Tropomyosin receptor kinase (Trk)
tropomyosin receptor kinase B (TrkB)
cAMP-dependent protein kinase (PKA)
cell adhesion molecules (CAMs)
tissue-type plasminogen activator (tPA)
urokinase plasminogen activator (uPA)
plasminogen activator inhibitor 1 (PAI-1)
vitronectin (VN)
low density lipoprotein-receptor related protein 1 (LRP-1)
matrix metalloproteinase (MMP)
uPA receptor (uPAR)
epidermal growth factor receptor (EGFR)
platelet-derived growth factor (PDGF)
tPA knockout (tPA−)
epidermal growth factor (EGF)
PDGF receptor (PDGFR)
middle cerebral artery occlusion (MCAO)
extracellular signal–regulated kinases (ERK)
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IkBα)
postsynaptic density protein 95 (PSD95)
amyloid precursor protein (APP)
tPA knockout (tPA−/−; B6.129S2-Plattm1Mlg/J)
light-dark (LD)
C57BL/6J wild-type (WT)
central nervous system (CNS)
Earle's Balanced Salt Solution (EBSS)
zeitgeber time (ZT)
Single unit activity (SUA)
lipopolysaccharide (LPS)
neural cell adhesion molecules (NCAMs),
uPA receptor (uPAR)
1 INTRODUCTION
1.1 Circadian Rhythms

1.1.1 Circadian timekeeping mechanisms and the SCN

To cope with life on this planet with its 24 hour (h) rotation, organisms have developed internal timekeeping mechanisms that allow them to predict and adapt to daily changes in the environment. These internal oscillatory systems generate approximately 24 h (circadian) cycles in behavior, physiology, and metabolism, enabling organisms to coordinate their activities with the environment. Nearly all organisms, ranging from single celled cyanobacteria to humans, exhibit circadian rhythms. These rhythms are present in a plethora of physiological processes, including metabolism, hormone secretion, and sleep-wake cycles. The ability to anticipate and respond to environmental changes, notably the light-dark cycle, is thought to confer evolutionary advantages, and disruptions of internal timekeeping mechanisms or asynchrony between the internal clock and the external environment result in severe health consequences, such as shift work disorder, increased risk for metabolic syndrome, and increased risk for certain cancers (reviewed in: (Bass & Takahashi, 2010; Gamble et al., 2014; Silver & Kriegsfeld, 2014; Man et al., 2016)).

Three characteristics distinguish circadian rhythms from other biological oscillations. First, circadian rhythms cycle endogenously in the absence of environmental input. In constant conditions, they cycle with an approximately 24 h period, a state known as free-running. Second, they are temperature compensated, meaning the period remains constant in a variety of physiologically relevant temperatures. And finally, circadian rhythms can synchronize to the environment, a process known as entrainment, by responding to environmental stimuli with a period close to 24 h that act as zeitgebers (Aschoff, 1960), or “time-givers.” Light serves as the dominant zeitgeber for most species, providing input that locks the endogenous clock to the daily light/dark cycle (reviewed in (Chaix et al., 2016; Hurley et al., 2016; Herzog et al., 2017).

In mammals, circadian rhythms are governed by a central circadian pacemaker, located in the suprachiasmatic nucleus (SCN) of the hypothalamus in the brain (Gamble et al., 2014; Silver & Kriegsfeld, 2014; Bass & Lazar, 2016; Cribbet et al., 2016). The SCN is a bilateral structure comprised of densely packed neurons and astroglial cells located dorsal to the optic chiasm, straddling the third ventricle. Most SCN neurons release the
neurotransmitter gamma-Aminobutyric acid (GABA), but the SCN is quite heterogeneous, and can broadly be divided into the retinoreceptive gastrin-releasing peptide (GRP) and vasoactive intestinal peptide (VIP) expressing ventrolateral core and vasopressin (AVP) expressing dorsomedial shell (Hattar et al., 2006; McNeill et al., 2011; Mohawk & Takahashi, 2011; Herzog et al., 2017). An elegant series of studies defined the SCN as the master circadian pacemaker. Early work suggested the clock may be found in the hypothalamus (Richter, 1965), followed by identifying the SCN as the terminal point for the RHT (Hendrickson et al., 1972; Moore & Eichler, 1972; Moore & Lenn, 1972; Moore, 1973). SCN lesion studies demonstrated that ablation of the SCN eliminates circadian rhythms (Moore & Eichler, 1972; Stephan & Zucker, 1972; Rusak, 1979). Metabolic imaging and electrophysiology demonstrated that the SCN exhibits circadian rhythms in vivo (Inouye & Kawamura, 1979), and 2-deoxyglucose (2-DG) radiolabeled imaging (Schwartz & Gainer, 1977) and electrophysiology on SCN brain slices demonstrated that the SCN retains this rhythmicity in vitro (Green & Gillette, 1982; Groos & Hendriks, 1982; Shibata et al., 1982). The strongest support of the SCN as the primary pacemaker came from studies demonstrating that transplantation of fetal SCN grafts into the brains of SCN ablated animals restores circadian rhythms, with a period identical to that of the host (Drucker-Colin et al., 1984; Sawaki et al., 1984; DeCoursey & Buggy, 1989). Finally, SCN cell culture studies demonstrated that circadian rhythms are cell autonomous (Welsh et al., 1995).

The current model for the SCN circadian oscillator consists of a cell autonomous transcriptional-translational-posttranslational negative feedback loop (TTFL) involving a group of clock genes that includes Period (Per) 1 and 2; Cryptochrome (Cry) 1 and 2, Bmal1, and Clock (Reviews: Hardin, 2004; Gallego & Virshup, 2007; Buhr & Takahashi, 2013; Hastings et al., 2014). BMAL1 and CLOCK proteins are transcriptional activators that act as a heterodimer to increase transcription of Per and Cry (Vitaterna et al., 1994; Reppert & Weaver, 2002). PER and CRY proteins accumulate in the cytoplasm, until they reach a critical level where PER and CRY proteins enter the nucleus, dimerize, and inhibit the activity of BMAL1/CLOCK. This suppresses Per and Cry transcription, and once PER and CRY levels decrease the cycle restarts. Phosphorylation of PER proteins leads to their degradation, which slows the rate of accumulation. Additional regulators of this core mechanism, including a redox oscillator and a cell membrane oscillator, are thought
to strengthen and stabilize the SCN circadian clock (Gillette & Wang, 2014; Hastings et al., 2014; Milev et al., 2015). The TTFL is found in every cell in the body, and it acts as the gears of the clock, generating and maintaining a ~24 h cycle. In the SCN, the rhythms of individual cells are coordinated to one another, then entrained to the environment, and the phase from the SCN is distributed to the remainder of the body.

Several stimuli can entrain the clock, creating a period equal to the entraining cycle. These include food availability (Edmonds & Adler, 1977), social contact (Mrosovsky, 1988; Mrosovsky et al., 2005), temperature, moonlight (Neumann, 1989; Fernandez-Duque & Erkert, 2006), or tides (a unique case allowing 24 h entrainment to an approximately 12 h input) (Palmer, 2000). However, light signals are the dominant synchronizing signal (Czeisler, 1995). The SCN receives input from the retina, intergeniculate leaflet (IGL), and raphe nuclei, but retinal signals are the primary source of photic phase-resetting information. Light pulses during subjective night or transitions from light to dark at subjective dawn or dusk shift the clock phase (Daan, 1977; Johnson, 1999; Johnson et al., 2003; Roenneberg et al., 2003). Depending on the time of day, phase shifts can either result in a shift forward or a fall back in clock timing. Light stimulates melanopsin containing retinal ganglion cells to release neurotransmitters (Johnson et al., 1989; Berson et al., 2002), including glutamate (Ebling, 1996; Hannibal, 2002), pituitary adenylate cyclase-activating peptide (PACAP) (Hannibal, 2006), and substance P onto SCN neurons (Chen et al., 1999; Golombek et al., 2003; Fahrenkrug, 2006; Hannibal, 2006). Of these, several lines of evidence support glutamate as the primary photic signal (Ding et al., 1994; Golombek & Rosenstein, 2010; Welsh et al., 2010). The SCN contains α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPA) and N-Methyl-D-aspartic acid receptors (NMDAR), but only NMDAR stimulation replicates phase shifting (Colwell & Menaker, 1992; Vindlacheruvu et al., 1992; Gannon & Rea, 1993; Ding et al., 1994; Gannon & Rea, 1994; Shirakawa & Moore, 1994b; a). Blocking NMDARs prevents phase shifts, suggesting that NMDARs are the primary source of glutamatergic phase shifting signals in the SCN (Ding et al., 1994). Activation of NMDARs results in a calcium influx (Ding et al., 1998; Obrietan et al., 1998; Colwell, 2000; 2001), which through the activation of a variety of downstream effectors (discussed below) results in a phase shift. Importantly, phase shifting only occurs when these events
happen during the subjective night-time, and not during the subjective day (Meijer & Schwartz, 2003; Cheng & Obrietan, 2006; Colwell, 2011).

The intracellular molecular mechanisms linking light stimuli to phase shifts are not fully elucidated, but several downstream effectors and signaling cascades have been demonstrated to play a role. A variety of kinases are activated following the NMDAR calcium influx. An early effect is activation of Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) by autophosphorylation on Thr286/287 (Fukushima et al., 1997; Yokota et al., 2001; Golombek et al., 2004). Increases in p(hosphorylated)CaMKII result in phosphorylation of neuronal nitric oxide synthase (nNOS), causing nitric oxide (NO) production (Agostino et al., 2004). Downstream of NO production the process of phase shifting bifurcates (Golombek et al., 2004). In the early night, phase delays require calcium-induced calcium release from ryanodine receptors (RyRs) (Ding et al., 1998). In the late night, NO activates guanylate cyclase (GC) and increases cyclic guanosine monophosphate (cGMP) (effects that do not happen in the early night), resulting in a phase advance (Weber et al., 1995; Ding et al., 1998; Prosser, 1998b; Tischkau et al., 2003). The phase delaying and phase advancing pathways re-converge, and there is a transient rapid phosphorylation of cAMP response element binding protein (CREB), that ultimately causes transcription of Per and other cAMP response element (CRE) regulated genes, effectively resetting the clock phase (Ginty et al., 1993; Ding et al., 1997; Gau et al., 2002). Also involved are mitogen-activated protein kinase (MAPK) and protein kinase A (PKA), but their link to entrainment isn’t well understood (Obrietan et al., 1998; Tischkau et al., 2000; Butcher et al., 2002; Antoun et al., 2012). Light-induced phase shifts can be mimicked \textit{in vitro} by application of glutamate to SCN brain slices (Golombek & Rosenstein, 2010; Iyer et al., 2014; Cooper, Submitted). \textit{In vivo} phase shifts can be assessed by observing behavioral patterns, and \textit{in vitro} phase shifts can be assessed by observing a variety of clock outputs, including rhythmic clock gene expression and neuronal activity patterns.

The intracellular oscillatory mechanisms described above create an elegant pacemaker model, but they do not fully account for several important SCN functions including synchronization and entrainment. Accumulating evidence suggests that extracellular molecules contribute to these processes. Intercellular coupling mechanisms within the SCN synchronize the individual cell’s rhythms to one another, and recent
evidence highlights an important role for astrocytes in maintaining SCN synchrony (Evans, 2016; Herzog et al., 2017). A variety of extracellular molecules are implicated in the process of phase shifting, including extracellular proteases and cell adhesion molecules such as neural cell adhesion molecule (NCAM), L1 cell adhesion molecule (L1-CAM), neurexins and neuroligins, Eph-ephrins, and integrins (Prosser et al., 2003; Mou et al., 2009b; Cooper, Submitted). Many of these extracellular molecules overlap with interactions on the extracellular matrix (ECM), which acts as a critical regulator of synaptic connections (Song & Dityatev, 2018). Many extracellular molecules also have astrocytic roles. The precise mechanisms through which all of these molecules work have not been fully elucidated yet, but it is apparent that changes in the extracellular space exert dramatic influence over the timekeeping mechanisms.

1.1.2 Neuronal plasticity in the SCN – daily rhythms in neuronal responses to glutamate

One particularly intriguing phenomenon in the SCN is a dramatic duality in responses to glutamate over the course of the day. Neuroplasticity refers to the ability of stimuli to induce structural and functional changes in neuronal responses. It is most thoroughly studied in terms of long term potentiation (LTP) and long term depression (LTD) in the hippocampus, where different patterns of neuronal stimulation induce either exaggerated (LTP) or attenuated (LTD) responses to subsequent stimuli (Malenka & Bear, 2004). The SCN exhibits a unique case of circadian plasticity where the response to a stimulus (light/glutamate), differs depending on the time of day that stimulus is presented (Iyer et al., 2014). As mentioned above, glutamate (or light) induces phase shifts at night, but not day, which means there is an endogenous rhythm in the neuronal responsiveness in the SCN (Iyer et al., 2014). This is further complicated by the divergent direction of shift in the early night vs late night. This suggests that there are persistent changes in synaptic connections that occur on a 24 h cycle, allowing for these cycles in neuronal responsiveness (Iyer et al., 2014). While much remains unclear about the mechanisms underlying this phenomenon, termed “daily iterative metaplasticity,” it is apparent that the mechanisms that influence neuronal plasticity in other systems, particularly the hippocampus, often have a conserved function of gating phase shifting in the SCN (Iyer et al., 2014). Some events that contribute to this daily plasticity include rhythms in
membrane potential, expression of proteins that influence neuroplasticity, and expression/functional status of intracellular signaling molecules (Iyer et al., 2014).

An important mediator of glutamate-induced phases shifts, NMDARs, are intricately associated with neuronal plasticity, and changes in their expression patterns, subunit composition, cell surface localization, and interacting partners can dramatically alter neuronal responses (Hunt & Castillo, 2012). NMDARs are heteromeric complexes containing four subunits generally composed of two obligate NR1 subunits complexed with a combination of NR2 (A-D) or NR3 (A-B) subunits (Moriyoshi et al., 1991; Traynelis et al., 2010; Paoletti et al., 2013; Iacobucci & Popescu, 2017). NMDAR subunit composition and phosphorylation patterns can influence their localization and activity patterns. NMDARs exhibit distinct diurnal rhythms in the SCN, with mRNA of ε3 (also known as NR2A) and ζ1 (NMDAR1) high during the day and low at night in rats, with anti-phase patterns in their respective proteins (Ishida et al., 1994). Expression of those two NMDARs also increases in response to light stimulation in the subjective night (Ishida et al., 1994). Expression and phosphorylation of NR2A and NR2B protein exhibits circadian rhythms in hamster SCN, with phosphorylated NR2B peaking in the late night (Wang et al., 2008). There are also endogenous rhythms in magnitude and duration of NMDAR calcium transients in SCN, which peak during the night, as does a rhythm in NMDAR-evoked currents (Pennartz et al., 2001). Collectively, these data suggest that NMDAR function peaks at night, which could allow increased responsiveness to glutamate. However, quite a bit remains unknown about actions of NMDARs as gating molecules in the SCN, and importantly, changes in cell surface localization and potential modulators of NMDAR function remain largely un-investigated.

Growth factors also contribute to the diurnal responses. Brain-derived neurotrophic factor (BDNF) is a secreted neurotrophin that regulates neuronal signaling across the central nervous system (CNS), and is important for a variety of neuroplastic events (Kowianski et al., 2018). BDNF exerts its function by binding to neurotrophin receptors, p75 and tyrosine kinase B receptors (TrkB) (Reichardt, 2006). In the nervous system, BDNF binding to TrkB is important for many of its effects on neuroplasticity. BNDF binding induces TrkB autophosphorylation, which activates a variety of protein kinases (Chao & Hempstead, 1995; Reichardt, 2006). BDNF is transcribed as an ~32 kDa proBDNF that is cleaved into ~14 kDa m(ature) BDNF, which is the form that binds TrkB
receptors (Foltran & Diaz, 2016). In the SCN, BDNF levels are rhythmic — they are high at night and low during the day (Liang et al., 1998). At night, these high levels of BDNF enable it to bind TrkB receptor, which in other regions can stimulate concurrent phosphorylation of NR1 NMDAR subunits (Slack et al., 2004) and in the SCN enables glutamate-induced phase shifts (Liang et al., 2000; Allen et al., 2005; Mou et al., 2009b). Inhibiting TrkB receptors or decreasing BDNF expression disrupts SCN responses to glutamate (Allen et al., 2005). Collectively, it seems that BDNF and TrkB receptors are key molecules acting to gate phase shifts, but similar to NMDARs, many questions still remain. In particular, we do not fully understand how they are regulated and respond to daily changes in the SCN.

The gating of photic phase resetting also involves intracellular signaling events, including the actions of cAMP. In the rat SCN, there are spontaneous oscillations in cAMP levels and cAMP-dependent protein kinase (PKA) activity (Prosser & Gillette, 1991). cAMP levels peak at the end of the day and the night, which corresponds to the day/night transitions (Prosser & Gillette, 1991). cAMP levels also increase in response to light or glutamate stimulation, but application of cAMP agonists does not mimic the phase shifting effects of light or glutamate stimulation (Tischkau et al., 2000). Concurrent activation of cAMP/PKA with light or glutamate results in enhanced phase shifts during the early night, but diminished phase shifts in the late night (Tischkau et al., 2000). Thus, the cAMP/PKA system may alter the baseline status of cell signaling pathways based on time of activation, resulting in opposing responses at different times (Tischkau et al., 2000). Similar changes in other intracellular effectors may also participate in gating photic phase shifts, but the molecules controlling their diurnal variations have not been elucidated.

In general, the circadian oscillator sets the cellular stage such that it is more responsive to photic stimuli at night than during the day (Iyer et al., 2014). As discussed above, several signaling molecules are known to be under circadian control, but these changes pale when compared to the complex picture of neuronal synaptic plasticity generally. Strengthening and weakening synaptic connections involves growth factors and their direct downstream effectors, changes in receptor expression, localization, and activation status, differences in astrocytic clearance of neurotransmitters from the synapse, adjustments in synaptic morphology, and ECM reorganization. Importantly, there is evidence that similar plasticity events may be happening in the SCN, particularly
with response to ECM changes. For example, there is evidence that a variety of ECM-associated proteins, such as cell adhesion molecules (CAMs) and extracellular proteases, are involved in circadian clock phase regulation (Cooper, Submitted). There are also data suggesting that the ECM may be important for daily changes in synaptic ultrastructure, which may be important for gating photic signals. There are day/night variations in glial and axonal terminal coverage on VIP neurons: during the night glia tightly surround VIP dendrites and are retracted from AVP dendrites, while in the day-time they move to cover AVP dendrites more closely and retract from VIP dendrites (Becquet et al., 2008; Girardet et al., 2010). Similar changes in other brain regions are restricted by the presence of the ECM and enabled following ECM remodeling. As summarized below, we have started identifying a few extracellular modulators of glutamate signals in the SCN, but much remains unknown about how the SCN achieves the day-night duality of responses.

1.2 The Plasminogen Activators

One particularly interesting group of proteins that modulates neuronal activity are extracellular proteases. A variety of secreted proteases exert dramatic influence over neuronal processing by cleaving ECM molecules, cell adhesion molecules, and growth factors, as well as through both proteolytic and non-proteolytic interactions with cell surface molecules (Salazar et al., 2016). These actions can facilitate structural changes in the extracellular space, change the strength of synapses, have direct effects on neurotransmitter receptors, and act as signaling events, all of which serve to mediate changes in cellular responses as a consequence of changes in the extracellular space (Salazar et al., 2016). The plasminogen activators, tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA), are extracellular proteases that modulate a variety of neuronal processes, and with particular relevance to circadian clock phase shifting, they have been found to influence glutamatergic signaling.

tPA and uPA are serine proteases whose primary function is to cleave plasminogen into proteolytically active plasmin. tPA was identified in the vascular system for its role in dissolving fibrin blood clots, while uPA was originally found in the urinary tract. The plasminogen activators have overlapping and divergent functions and partners (Camiolo et al., 1971; Collen & Lijnen, 1991). Their activity is modified by inhibitors and interacting proteins, and they cleave a variety of downstream targets (Al-Horani, 2014).
tPA is secreted in a pro form, which can be cleaved by plasmin into a two-chain form (Chevilley et al., 2015). Both forms of tPA are proteolytically active, but differ in stability and targets (Chevilley et al., 2015). tPA activity is inhibited by two main inhibitors, plasminogen activator inhibitor 1 (PAI-1), which requires an interacting partner, vitronectin (VN), and neuroserpin, which is the primary inhibitor found in the brain (Al-Horani, 2014). tPA also interacts with several membrane-associated proteins, including low density lipoprotein-receptor related protein 1 (LRP-1) and annexin II (Archinti et al., 2011; Chevilley et al., 2015). Aside from activating plasminogen, tPA also cleaves ECM molecules, matrix metalloproteinases (MMPs), and CAMs (Archinti et al., 2011). uPA is also secreted in a single chain pro-form, but it remains inactive until it is cleaved by plasmin into the active two-chain form upon binding to its receptor, uPA receptor (uPAR) (Lijnen et al., 1987a). Similar to tPA, uPA is also inhibited by PAI-1 and neuroserpin, interacts with LRP-1 and annexin II, and it’s activity can affect ECM molecules, including MMPs and CAMs (Ishida et al., 1994; Archinti et al., 2011; Chevilley et al., 2015).

1.2.1 tPA regulates glutamate signaling in the brain

Early work suggested fibrinolytic activity occurs in the brain, and eventually supported tPA expression in the central nervous system (Fantl & Fitzpatrick, 1950; Takashima et al., 1969; Tovi, 1973; Krystosek & Seeds, 1981; Soreq & Miskin, 1981; Basham & Seeds, 2001). We now know that tPA, uPA, PAI-1, neuroserpin, and plasminogen are expressed in the brain and can be found in neurons and astrocytes of many regions, including the cortex, limbic system, hypothalamus, and cerebellum (Kalderon et al., 1990; Presta et al., 1990; Masos & Miskin, 1996; Lee et al., 2017). Subsequent research, discussed below, has demonstrated that the plasminogen activators and their interacting partners are potent regulators of neuroplasticity throughout the central nervous system.

tPA has received much attention for its diverse and complex roles in regulating neuronal processes. tPA is abundantly expressed in the brain, and can be found in endothelial cells (Sappino et al., 1993), glial cells (Siao et al., 2003), and neurons (Nicole et al., 2001; Yepes et al., 2009). In neurons tPA can be localized to synaptosomes (Zisapel et al., 1982). tPA expression is also inducible in the brain, and it has been identified as one of 5 immediate-early genes induced following seizure or high frequency
stimulation, suggesting tPA could modulate “structural changes that accompany activity-dependent plasticity” (Qian et al., 1993). tPA has been associated with many functions of the brain, including cell migration, neurite growth, cell-cell adhesion, synaptic plasticity, neurodegeneration, neuroprotection, neurovascular permeability, and Alzheimer’s disease (Lee et al., 2015). The mechanisms through which tPA functions are equally diverse. In some cases, tPA acts through plasmin-dependent functions. In other cases, tPA proteolytic activity that is independent of plasmin generation is important. Additionally, tPA can act through non-proteolytic mechanisms that often involve interactions with specific receptors, including annexin II, LRP-1, or NMDAR. (Qian et al., 1993; Madani et al., 1999; Oray et al., 2004; Yepes et al., 2016).

Evidence supporting a role for tPA in neuroplasticity comes from studies identifying involvement of tPA in LTP and LTD (Baranes et al., 1998; Fiumelli et al., 1999; Pang & Lu, 2004; Pang et al., 2004; Salazar et al., 2016; Medcalf, 2017). There are deficits in LTP in tPA knockout mice, and increases in LTP when tPA is over-expressed (Baranes et al., 1998; Pawlak et al., 2002). tPA enhances hippocampal learning, LTP, and LTP-associated synapse formation, and these effects are inhibited by PAI-1 (Baranes et al., 1998). tPA is also implicated in various non-hippocampal learning and memory paradigms (Melchor & Strickland, 2005). One mechanism through which tPA influences neuronal activity is by activating BDNF downstream of plasmin generation (Fiumelli et al., 1999; Pang & Lu, 2004; Pang et al., 2004). As discussed before, BDNF activation of TrkB influences neuronal responses to a variety of stimuli, and it also results in an increase in tPA expression (Kuzniewska et al., 2013). This creates the potential for a positive feed-forward loop that serves to increase tPA-dependent events. tPA, plasminogen, and pro-BDNF are co-localized within dense core granules of embryonic rat hippocampal neurons, these vesicles are transported preferentially to active dendritic spines, and the three proteins are co-secreted in response to LTP-inducing stimulation (Lochner et al., 2008). Thus, tPA modulation of BDNF signaling may be important for tPA-dependent effects on neuroplasticity throughout the brain.

Apart from activating BDNF, tPA has additional signaling targets in the brain. For example, tPA interacts with NMDAR NR1 and NR2B subunits and influences NMDAR signaling through a process that may involve plasmin-independent proteolytic cleavage of NR1, as well as through cleaving NR2A subunits via plasmin (Yuan et al., 2009; Ng et al.,
2012; Obiang et al., 2012). These actions affect NMDAR signaling, and generally act to increase the responsiveness and/or calcium influx in response to glutamate stimulation (Pawlak et al., 2005a; Pawlak et al., 2005b; Norris & Strickland, 2007), though tPA has been found to decrease responses to low NMDA concentrations in hippocampal neurons (Martin et al., 2008). tPA’s interactions with other receptors, including LRP-1 (discussed below), also contribute to hippocampal LTP (Zhuo et al., 2000). Another tPA-binding receptor, annexin II, is widely expressed throughout the brain, particularly in endothelial cells, and is thought to increase plasmin generation following tPA treatments (Kang et al., 1999; Zhao & Lu, 2007). Binding to these receptors can restrict tPA’s sphere of activity and modify its proteolytic activity, potentially serving as a co-receptor to increase proteolytic efficiency (Miles & Parmer, 2013; Chevillet et al., 2015). tPA interactions with growth factor pathways, such as interactions with epidermal growth factor receptor (EGFR) and platelet-derived growth factor (PDGF) also influence neuronal function (Zhuo et al., 2000; Miles & Parmer, 2013; Chevillet et al., 2015; Lemarchand et al., 2016). Collectively, through a combination of mechanisms, tPA modulates neuronal responses to such an extent that it has been characterized as a neuromodulator and potentially a gliotransmitter (Casse et al., 2012b).

1.2.2 uPA influences neuronal processes

uPA has not received as much attention as tPA in the central nervous system, but it also participates in synaptic remodeling, particularly during development and following injury (Merino et al., 2017a). uPA and uPAR are highly expressed in the CNS during development, and can be found in neurons, microglia, and astrocytes (Cho et al., 2012; Wu et al., 2014; Merino et al., 2017a). uPA and uPAR levels are very low in the adult brain, but they increase following injury (Wu et al., 2014). uPA, uPAR, and LRP-1 are rapidly upregulated following spinal cord hemisection, followed by an increase in tPA (Seeds et al., 2009). uPA acting via uPAR is necessary for post-hemisection remodeling (Seeds et al., 2009). Additionally, following ischemic stroke, uPA-uPAR signaling enhances dendritic spine recovery in cortical neurons (Wu et al., 2014). The evidence supports a model where neurons release uPA following oxygen glucose deprivation, which binds astrocytic uPAR to allow synaptic recovery (Diaz et al., 2017). In this system, uPA may be acting to activate astrocytes, as uPA induces GFAP expression (a marker of
astrocytic activation) in wildtype but not uPAR knockout mice (Diaz et al., 2017). uPA has also been reported to cleave NR1 and NR2B subunits to alter NMDAR signaling (Ng et al., 2012). uPAR knockout mice exhibit disrupted neuronal networks, with fewer cortical GABAergic interneurons, increased seizures, enhanced anxiety, and decreased social interactions (Powell et al., 2003; Eagleson et al., 2005; Ndode-Ekane & Pitkanen, 2013; Wu et al., 2014; Rantala et al., 2015). uPAR interacts with cell-surface proteins, including LRP-1, integrins, and receptor tyrosine kinases, and these interactions often mediate internalization of the receptor-protein-ligand complex (Madsen et al., 2007; Miles & Parmer, 2013; Van Gool et al., 2015). Although uPA has not been investigated as extensively as tPA in regards to neuronal processes, it has been well studied in the context of cancer, including glioblastoma, and mechanistic insights from pathological situations may provide clues to its physiological roles (Mohanam et al., 1994; Rustamzadeh et al., 2003). It is important to note that while current evidence implicates tPA as a modulator of neuroplasticity and focuses on uPA in the context of injury response, we cannot assume that they don’t overlap in these functions. Indeed, one protease may compensate for the other in a variety of contexts, and untangling the associations and differences between the two will be important to our understanding of the plasminogen activators.

1.2.3 tPA regulates glutamate-induced phase shifts

Because of the overlap between tPA’s role in the CNS and processes already known to gate glutamate-induced phase shifting, particularly regarding BDNF maturation, our lab undertook a study of tPA in the mammalian SCN (Mou et al., 2009b). We found that tPA contributes to the processes regulating glutamate-induced phase shifting of the SCN circadian clock in vitro, identifying a new role for plasminogen activators in the brain. Members of the plasminogen activating cascade, including PAI-1, VN, tPA, plasminogen, plasmin, mBDNF, and proBDNF, are all expressed in the SCN (Mou et al., 2009b). tPA, plasmin, and mBDNF expression exhibit diurnal rhythms, with higher levels at night than during the day, while PAI-1 exhibits an inverse rhythm with high daytime expression (Mou, 2010; Cooper et al., 2017). Treating SCN slices in vitro with PAI-1 blocks glutamate-induced phase delays at ZT16 and phase advances at ZT23, indicating that tPA proteolytic activity is necessary for these phase shifts (Mou et al., 2009b). Additional evidence supports a role for tPA acting upstream of BDNF to gate clock phase shifts: inhibiting
plasmin with α2-antiplasmin blocks glutamate-induced phase shifts, and co-application of plasmin or mBDNF but not plasminogen recover phase shifting (Mou et al., 2009b). Vitronectin is also necessary for PAI-1’s actions in the SCN, as PAI-1 does not block phase shifts in vitronectin knockout mice (Mou et al., 2009b). Collectively, this suggests that at night, when tPA levels are highest, it converts plasminogen into plasmin, which then cleaves proBDNF into mBDNF, which binds to TrkB receptors enabling glutamate-induced phase shifts (Mou et al., 2009b).

Glutamate-induced changes in tPA expression could contribute to tPA’s role in phase shifting, and thus Mou and colleagues also investigated changes in protein expression following glutamate treatment (Mou, 2010). Glutamate application to in vitro SCN slices increases tPA levels in the early night, but not in the late night or mid-day. This glutamate treatment does not alter PAI-1, plasmin, or BDNF levels (Mou, 2010). Treating SCN slices with glutamate and PAI-1 concurrently causes a reduction in plasmin, while concurrent glutamate and α2-antiplasmin reduces pro- and mBDNF levels (Mou, 2010). And finally, mBDNF levels are lower in tPA knockout (tPA−/−) mice when compared to wild-type (WT), supporting tPA as a mediator of BDNF maturation (Mou, 2010). Collectively these data generally support the model described above. However, a couple of discrepancies suggest that tPA’s role in the SCN may be more complex. First, the glutamate-induced increase in tPA expression in the early night but not in the late night are at odds with the finding that PAI-1 prevents phase shifts at both times, which may mean there are mechanistic differences between early and late night phase shifts. Second, it is interesting that α2-antiplasmin reduces both pro- and mBDNF levels, as this suggests it is regulating total BDNF expression rather than just BDNF maturation.

This work defined a role for tPA acting upstream of mBDNF to gate glutamate phase resetting responses in the SCN, but also created a variety of questions. This study did not investigate circadian phase shifting in tPA knockout mice either in vivo or in vitro. Whether or not uPA is involved in circadian clock phase regulation also was not addressed. Additionally, tPA does not work in isolation, and some key potential contributors to tPA function in the SCN remained uninvestigated. In particular, LRP-1 mediates a variety of tPA-dependent functions in the brain and periphery, and therefore could be influencing the circadian clock. tPA has also been reported to modulate neuronal signaling through direct interactions with NMDARs, and this possibility was not
investigated with respect to SCN clock phase regulation. The work presented here addresses these knowledge gaps in two ways. First, we assessed SCN phase shifting in tPA<sup>-</sup> mice, and uncovered an unexpected role for uPA in regulating clock phase shifting. Second, we investigated LRP-1 as a potential modulator of SCN phase shifts, with a specific focus on interactions between LRP-1, tPA, and NMDARs. My aims were twofold: first, to increase our understanding of how extracellular molecules influence synaptic plasticity in the SCN by continuing our investigation of extracellular proteases; and second, to investigate the closely associated membrane receptor, LRP-1, whose activity is associated with transducing information regarding the extracellular space and responding to changes in ligands by mediating their endocytosis and/or activating signal transduction. Together, this research furthers our understanding of the mechanisms gating SCN circadian clock phase shifting, and simultaneously advances our knowledge of how this group of extracellular synaptic plasticity modulators functions in the brain.

### 1.3 Low-density lipoprotein receptor-related protein-1

#### 1.3.1 LRP-1 introduction

LRP-1, a member of the low-density lipoprotein (LDL) receptor family, is a large membrane receptor that is widely expressed in many tissues. LRP-1 is 600 kDa protein composed of a 515 kDa extracellular αLRP-1 subunit that is non-covalently associated with an 85 kDa transmembrane βLRP-1 subunit (Kerrisk et al., 2014; Ramanathan et al., 2015). The αLRP-1 subunit contains cysteine-rich complement-type ligand binding repeats, and epidermal growth factor (EGF) repeats and β-propeller domains that function in the release of ligands (Lillis et al., 2005). The βLRP-1 subunit contains a single transmembrane domain and cytoplasmic domain, which has two NPxY motifs that can be phosphorylated to contribute to endocytosis and signal transduction (Lillis et al., 2005). Additionally, the extracellular subunit can be "shed" via metalloproteinase cleavage on the β subunit, generating a soluble protein whose function isn’t entirely understood, but may serve to sequester and inactivate LRP-1 ligands in the extracellular space (Van Gool et al., 2015).

LRP-1 is a highly efficient transport protein, which binds over 50 different ligands extracellularly, and can mediate rapid endocytosis or influence signal transduction
following ligand binding (Lillis et al., 2005). Through these functions, it regulates a variety of physiological processes, including lipoprotein metabolism, protease degradation, lysosomal enzyme activation, and cellular entry of bacterial toxins and viruses (Lillis et al., 2008). LRP-1 ligands are diverse, and include APO-E, tumor growth factor-β, MMP’s, neuroserpin, tPA, uPA, and amyloid-β (Lillis et al., 2005). LRP-1 also interacts with a variety of cytoplasmic adaptor proteins in a phosphorylation-specific manner, and can modulate activity of transmembrane receptors such as integrins and receptor tyrosine kinases (Lillis et al., 2005). Regulation of signal transduction is often coupled with other cell surface receptors such as PDGF receptor (PDGFR) and leptin receptors, and mediated via intracellular kinases (Muratoglu et al., 2010; Liu et al., 2011; Strickland et al., 2014). Interactions with other receptors, including uPARs and NMDARs have also been shown to mediate LRP-1 dependent effects (Lillis et al., 2005; Ramanathan et al., 2015; Van Gool et al., 2015).

1.3.2 LRP-1 regulates neuroplasticity throughout the brain

LRP-1 is abundantly expressed in the central nervous system, where it can be found on vascular smooth muscle cells, pericytes, astrocytes, and neurons (Lillis et al., 2008; Shinohara et al., 2017). Deletion of the Lrp1 gene is embryonically lethal, indicating a critical involvement in development (Lillis et al., 2008; Liu et al., 2010). Neuronal specific deletion of Lrp1 in mice results in severe behavioral and motor abnormalities, including hyperactivity, tremor, and dystonia (May et al., 2004). LRP-1 protein can be expressed in the post synapse, where it regulates synaptic integrity, partly through regulating glutamate receptors (May et al., 2004).

tPA is a prominent LRP-1 ligand, and tPA binding to LRP-1 activates LRP-1 dependent signaling in a variety of contexts (Zhuo et al., 2000; Yepes et al., 2003; Samson et al., 2008; Echeverry et al., 2010). In rat kidney interstitial fibroblasts, tPA acts as a cytokine by binding to LRP-1, inducing tyrosine phosphorylation on LRP-1’s intracellular domain, and triggering signal transduction that is able to induce specific gene expression, including Mmp9 expression (Hu et al., 2006). In this system, tPA stimulates extracellular signal–regulated kinases (ERK1/2) phosphorylation to protect against apoptosis, and LRP-1 is required for these events (Hu et al., 2008). tPA can also stimulate ERK1/2 activity in hippocampal neuronal cell culture, and LRP-1 is necessary for this event (Martin
et al., 2008). The results of this study support tPA, NMDAR, and LRP-1 acting in complex to mediate tPA-dependent effects, and identified the distal NPxY motif on LRP-1 as a key mediator of the LRP-1/NMDAR interaction (Martin et al., 2008). Additionally, LRP-1 mediates the LTP-enhancing effect of tPA in tPA⁻⁻ hippocampal slices (Zhuo et al., 2000). LRP-1 is also required for tPA-mediated microglial activation in the central nervous system (CNS) following middle cerebral artery occlusion (MCAO; a model of brain ischemia/stroke) (Zhang et al., 2009b). tPA increases MMP-1 expression following MCAO, and this also depends on the presence of LRP-1 (Zhang et al., 2009a). In cultured Schwann cells LRP-1 functions as an injury detection receptor by inducing c-Jun phosphorylation downstream of tPA binding (Flutsch et al., 2016). Astrocytic-derived tPA induces astrocytic outgrowth via LRP-1-dependent induction of ERK activity (Qian et al., 2016). tPA and LRP-1 have complementary effects on lipopolysaccharide (LPS)-induced inflammation, where tPA inhibits LPS induced inflammation through a pathway that involves LRP-1 (Mantuano et al., 2017). Collectively, these studies are consistent with the idea that tPA binding to LRP-1 can initiate a variety of signaling events. In addition to its signaling responses, LRP-1 can mediate the clearance of tPA by transporting tPA across the blood-brain barrier (Benchenane et al., 2005). LRP-1 also contributes to tPA recycling in the nervous system (Casse et al., 2012b): glutamate induces LRP-1 dependent astrocytic endocytosis and recycling of tPA (Casse et al., 2012b). And finally, there is evidence that LRP-1 may act as a co-receptor, increasing tPA proteolytic activity, as it does for tPA-mediated cleavage of platelet derived growth factor-CC (PDGFR-CC) (Su et al., 2017). Thus, the interactions between tPA and LRP-1 in the nervous system are complex and multifunctional.

LRP-1 can also modulate neuronal activity through its association with NMDA receptors. LRP-1 physically interacts with NMDAR via binding to postsynaptic density protein 95 (PSD95) (May et al., 2004), allowing it to control NMDAR trafficking and degradation, and thus NMDAR surface localization (Maier et al., 2013). LRP-1 modulates neuronal calcium signaling via NMDAR (Bacskai et al., 2000). LRP-1’s intracellular domain can regulate NMDAR-mediated signaling processes (Nakajima 2013), and LRP-1 can form complexes with Trk receptors to modulate NMDAR signaling (Mantuano et al., 2013). As mentioned above, LRP-1 may also influence NMDAR signaling by regulating the activity of tPA (Casse et al., 2012b). Separately, LRP-1 can influence neuronal...
plasticity through interactions AMPA receptors. LRP-1 has been found to influence the trafficking, phosphorylation, and turnover of GluA1 subunits of AMPA receptors (Gan et al., 2014). One final association worth mentioning involves interactions between LRP-1 and amyloid precursor protein (APP). LRP-1 binds and mediates cellular catabolism of longer forms of APP (Kounnas et al., 1995), and internalizes transmembrane isoforms of APP (Knauer et al., 1996). LRP-1 mediated internalization of both soluble and membrane forms of APP may lead to changes in neuronal activity. In general, it seems that in addition to its endocytic and signaling activities, LRP-1 functions to regulate surface localization of a variety of membrane receptors.

1.3.3 **LRP-1 overlaps with mechanisms that gate phase shifts**

LRP-1 acts as a sensor and regulator of the extracellular space, and responds to extracellular changes in ways that modulate neuronal responses. The aforementioned functions of LRP-1 all intersect with processes known to gate SCN clock phase shifting, and in particular they influence responses to glutamate signals. LRP-1 regulates and responds to extracellular tPA, which is an important gatekeeper for glutamate-induced phase shifting. NMDARs are also important mediators of glutamate signals in the SCN, and LRP-1 can influence NMDAR activity. And finally, TrkB receptors act to gate phase shifts, and LRP-1-Trk receptor complex formation further strengthens the links between LRP-1 and known clock regulators. Given these close associations, I hypothesized that LRP-1 contributes to circadian clock phase regulation. Furthermore, interactions between LRP-1 and the plasminogen activating cascade, or interactions with NMDARs may underlie LRP-1s role in the clock. To the best of my knowledge, no studies had assessed LRP-1 involvement in circadian clock phase shifting prior to my research.
2 UROKINASE-TYPE PLASMINOGEN ACTIVATOR MODULATES MAMMALIAN CIRCADIAN CLOCK PHASE REGULATION IN TISSUE-TYPE PLASMINOGEN ACTIVATOR KNOCKOUT MICE
A version of this chapter was originally published by Joanna M. Cooper, Ashutosh Rastogi, Jessica A. Krizo, Eric M. Mintz, and Rebecca A. Prosser:


This article is presented in its as-published form. Joanna Cooper was responsible for the conceptualization, experiment design, performing the in vitro electrophysiology experiments, western blotting, and casein-plasminogen gel zymography experiments, and writing. Ashutosh Rastogi, a postdoctoral fellow in the laboratory of Dr. Eric Mintz at Kent State University, performed, analyzed, and wrote the results for the in-vivo experiments with the help of Jessica Krizo, a graduate student in the same lab.

2.1 Abstract

Glutamate phase shifts the circadian clock in the mammalian suprachiasmatic nucleus (SCN) by activating NMDA receptors. Tissue-type plasminogen activator (tPA) gates phase shifts by activating plasmin to generate m(ature) BDNF, which binds TrkB receptors allowing clock phase shifts. Here, we investigate phase shifting in tPA knockout (tPA−/−; B6.129S2-Plattm1Mlg/J) mice, and identify urokinase-type plasminogen activator (uPA) as an additional circadian clock regulator. Behavioral activity rhythms in tPA−/− mice entrain to a light-dark (LD) cycle and phase shift in response to nocturnal light pulses with no apparent loss in sensitivity. When the LD cycle is inverted, tPA−/− mice take significantly longer to entrain than C57BL/6J wild-type (WT) mice. SCN brain slices from tPA−/− mice exhibit entrained neuronal activity rhythms and phase shift in response to nocturnal glutamate with no change in dose-dependency. Pre-treating slices with the tPA/uPA inhibitor, plasminogen activator inhibitor-1 (PAI-1), inhibits glutamate-induced phase delays in tPA−/− slices. Selective inhibition of uPA with UK122 prevents glutamate-induced phase resetting in tPA−/− but not WT SCN slices. tPA expression is higher at night than the day in WT SCN, while uPA expression remains constant in WT and tPA−/− slices. Casein-plasminogen zymography reveals that neither tPA nor uPA total proteolytic activity is
under circadian control in WT or tPA−/− SCN. Finally, tPA−/− SCN tissue has lower mBDNF levels than WT tissue, while UK122 does not affect mBDNF levels in either strain. Together, these results suggest that either tPA or uPA can support photic/glutamatergic phase shifts of the SCN circadian clock, possibly acting through distinct mechanisms.

2.2 Introduction

In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus is the primary circadian pacemaker, maintaining and synchronizing the daily rhythms of diverse physiological and behavioral processes (Moore & Eichler, 1972; Stephan & Zucker, 1972). The SCN exhibits self-sustaining oscillations in neuronal activity patterns that synchronize to the environment primarily through entrainment to light stimuli, a process known as photic entrainment. Photic entrainment depends on glutamate release onto SCN neurons inducing shifts in the phase of the underlying clock (Liou et al., 1986; Ding et al., 1994; Porterfield et al., 2007; Porterfield & Mintz, 2009).

Glutamate binds to NMDA receptors (NMDAR) to initiate a calcium influx and activate nitric oxide synthase. This stimulates protein kinases and transcriptional regulators to reset the core clock mechanism by altering the transcription of clock-associated genes (Ding et al., 1997; Gillette & Tischkau, 1999; Butcher et al., 2003; Pizzio et al., 2003; Butcher et al., 2004; Marpegan et al., 2004; Butcher et al., 2005). Light-induced phase shifts require concurrent activation of tropomyosin receptor kinase B (TrkB) via brain-derived neurotrophic factor (BDNF) binding. TrkB gates phase shifts through mechanisms that remain unclear, allowing them to only occur at night (Liang et al., 1998; Liang et al., 2000). The proteolytic conversion of proBDNF to m(ature)BDNF, mediated by plasmin, regulates BDNF signaling (Plow et al., 1995; Lee et al., 2001; Mou et al., 2009b).

The plasminogen activating system consists of various proteases, their inhibitors, and their receptors that collectively control the activation of plasminogen into plasmin (Saksela & Rifkin, 1988; Plow et al., 1995; Cesaran-Maus & Hajjar, 2005; Collen & Lijnen, 2005). Members of this system, including tissue-type plasminogen activator (tPA), urokinase-type plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1), urokinase plasminogen activator receptor (uPAR), neuroserpin, and plasminogen modulate neuronal processes throughout the brain (Samson & Medcalf, 2006). Of these,
tPA has received the most attention within the central nervous system (CNS) and its role as a neuromodulator is well established (Soreq & Miskin, 1981; Sappino et al., 1993; Fernandez-Monreal et al., 2004; Melchor & Strickland, 2005; Samson & Medcalf, 2006; Casse et al., 2012a). In the hippocampus, tPA proteolytic activity converts plasminogen into plasmin, and plasmin can exert downstream effects on synaptic plasticity by influencing extracellular matrix remodeling or by proteolytically generating mBDNF from proBDNF (Pang & Lu, 2004; Pang et al., 2004; Melchor & Strickland, 2005). There are also BDNF-independent and plasminogen-independent synaptic effects of tPA. For example, tPA can influence neuronal activity by modulating NMDAR activity and through interactions with specific receptors including low density lipoprotein receptor-related protein 1 (LRP-1) and annexin II (Nicole et al., 2001; Fernandez-Monreal et al., 2004; Zhang et al., 2009b; Baron et al., 2010; Jullienne et al., 2011). tPA’s activity is carefully controlled by its inhibitor, PAI-1, and the PAI-1 stabilizing protein vitronectin (Schleef et al., 1991; Delegue et al., 1998; Huntington & Carrell, 2001; Minor & Peterson, 2002; Mayasundari et al., 2004).

uPA also converts plasminogen into plasmin, is inhibited by PAI-1, is expressed in the CNS, and influences a variety of neuronal processes including Schwann cell migration, nerve regeneration, epilepsy, dendritic spine recovery following stroke, and amphetamine and morphine-induced reward (Ploug & Kjeldgaard, 1956; Hayden & Seeds, 1996; Iyer et al., 2014; Wu et al., 2014; Karagyaur et al., 2015). However, much remains unknown about the specific mechanisms of uPA’s involvement in modulating neuronal responses (Soleman et al., 2013; Chang et al., 2014; Katic et al., 2014; Lino et al., 2014; Wu et al., 2014). Importantly, little has been done to characterize the interactions between tPA and uPA in neuronal systems (Bahi & Dreyer, 2008).

The plasminogen activating system regulates circadian phase resetting in the SCN (Mou et al., 2009b). Using PAI-1 as a tPA inhibitor, our previous study showed that tPA proteolytic activity gates glutamate-induced phase shifting by generating plasmin, which cleaves proBDNF into mBDNF, which then binds to the TrkB receptor to allow glutamate to induce a phase shift (Mou et al., 2009b). Here, we investigate phase shifting in tPA−/− mice and shift our attention to possible involvement of uPA in circadian clock regulation.
2.3 Materials and Methods

2.3.1 Animals

All experiments used C57BL/6J wildtype (WT) mice from Harlan Labs or Jackson Laboratory, or tPA knockout (B6.129S2-Plat<sup>tm1Mlg</sup>/J; tPA<sup>−/−</sup>) mice. Male and female tPA<sup>−/−</sup> mice were generated at and purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). The tPA<sup>−/−</sup> mice were fully backcrossed for at least 8 generations into the same background C57BL/6J strain as WT, and the C57BL/6J strain is the recommended control for the tPA<sup>−/−</sup> mice. WT and tPA<sup>−/−</sup> mice were bred and group-housed in the Kent State University animal facility and the University of Tennessee Knoxville animal facility in a 12h-light/12h-dark cycle (12L:12D) and fed ad libitum. Male (in vivo and in vitro experiments) and female (in vivo experiments) mice aged 6-12 weeks of age at the beginning of each experiment were used, and the animals used for each study were age-matched as closely as possible. Experiments were conducted in accordance with Kent State University Institutional Animal Care and Use Committee (in vivo work) and the University of Tennessee Knoxville Institutional Animal Care and Use Committee (in vitro work).

2.3.2 In vivo methods

**Entrainment**

Six tPA<sup>−/−</sup> and six WT mice were utilized to examine the rate of entrainment to a shifted LD cycle. After at least two weeks of baseline activity measurement, the light cycle was inverted (shifted by 12 hrs). Activity patterns were monitored until all animals showed stable entrainment to the shifted LD cycle. Entrainment was defined as the date when the activity onset no longer shifted further on the subsequent day, and when the offset of activity was no longer showing further progression towards the time of light onset. The number of days until a stable phase of activity onset was reestablished was measured for each animal. Differences between genotypes were assessed using a two-sample t-test.

**Phase shifting response to light pulses**

Animals were individually housed in constant dark for at least 10 days with free access to running wheels and activity was monitored using Clocklab software. tPA<sup>−/−</sup> and WT mice were given a light pulse of either 300, 50, or 5 lux for 15 min at CT16 or 300 lux
at CT22, determined by activity onset defined as CT12 (n=3-6). Light intensities were measured at the cage bottom using a lux meter, and each animal was exposed to only one light pulse. Phase shifts were calculated using ClockLab software. This software utilizes a linear regression method proposed by Daan and Pittendrigh (Daan & Pittendrigh, 1976). A line was fit to activity onsets 10 days prior to the light pulse. A second line was fit to activity onsets 4-10 days after light pulse. Days 1-3 after the light pulse were not included in the data analysis. The phase shift was equal to the difference between the two regression lines.

**Free running periods**

Six WT and six tPA<sup>−/−</sup> mice were housed on a LD cycle, then transferred to DD for three weeks, LL for three weeks, and DD for three weeks again. Free running period was assessed in the last 10 days of each condition with Chi-squared periodograms.

### 2.3.3 *In vitro* methods

**Brain slice preparation**

Coronal brain slices (500 μm) containing the SCN were prepared from brains dissected following rapid decapitation of unanaesthetized adult male (>8 weeks old) WT or tPA<sup>−/−</sup> mice, housed in a 12:12 LD cycle. Slices were maintained as previously described (Prosser et al., 2003; Yamada and Prosser, 2014) in a Hatton-style brain slice dish, perfused constantly with oxygenated Earle’s Balanced Salt Solution (EBSS; Sigma-Aldrich) supplemented with glucose, bicarbonate, and gentamicin (pH 7.4) at 37˚ C. One mouse was used for each experiment and replicate experiments were performed on different mice.

**Slice treatments**

Drug treatments were bath applied on the first day *in vitro* following previously established protocols (Prosser et al., 2003; Yamada and Prosser, 2014). At ZT16 or ZT23 (Where ZT0 = lights-on in the donor animal colony, and ZT12 = lights-off) perfusion was stopped and the medium in the slice chamber was replaced with EBSS medium supplemented with PAI-1 (5 nM; Molecular Innovations Novi, MI, USA), uPA inhibitor (200 nM; UK122 from Santa Cruz, Dallas, TX, USA), and/or glutamate (1 μM – 1 mM; Sigma-Aldrich). For blocking experiments, slices were pretreated for 30 minutes with inhibitor
only (PAI-1 or UK122) starting at ZT15.5 (phase delays) or ZT 22.5 (phase advances), followed by 10 min treatment with 1 mM Glutamate + inhibitor. After 10 minutes the medium was replaced with normal medium and perfusion was reinstated. Glutamate alone treatments were applied for 10 minutes. PAI-1 or UK122 alone treatments were applied for 40 minutes starting at ZT15.5.

**Single unit activity (SUA) recordings**

Extracellular, single-cell recordings of neuronal activity were made on day 2 *in vitro*, using methods previously described (Prosser *et al.*, 1994a; Prosser *et al.*, 1994c; Ding *et al.*, 1997; Prosser, 1998a; Soscia & Harrington, 2004; Tischkau *et al.*, 2004). A glass micropipette containing 3M NaCl was lowered into the SCN until the signal from an individual neuron was isolated. The cell's activity was recorded for 5 minutes, after which the electrode was moved to find a new cell. Data acquisition and analysis were done using the computer program DataWave (DataWave Technologies, Loveland, CO, USA). Neuronal activity was sampled in this way for 10 hours (h). The firing rates of individual cells were then grouped into 2 h running means. Time of peak activity was determined as the time of symmetrically highest activity. The difference in time-of-peak of untreated slices vs. drug-treated slices was calculated to determine phase shifts. A minimum of 3 biological replicates were run for each experimental condition.

**2.3.4 Western blots**

Slices containing the SCN were maintained as described above then collected and immediately frozen at ZT6, ZT16, and ZT23. To control the amount of time slices were maintained *in vitro*, the following slicing and collecting paradigm was used: slices collected at ZT6 on day 1 *in vitro* were made at ZT2 (time *in vitro* = 4 h); slices collected at ZT16 on day 1 *in vitro* were made at ZT8 (time *in vitro* = 8 h); slices collected at ZT23 on day 1 *in vitro* were made at ZT3 (time *in vitro* = 20 h); and slices collected at ZT6 on day 2 *in vitro* were made at ZT10 (time *in vitro* = 20 h). SCN of two mice were pooled for each sample. For mBDNF experiments, WT and tPA^-/-* SCN slices were either left untreated or incubated with 200 nM UK122 for 40 minutes starting at ZT15.5. Slices were stored at -80˚C until protein extraction. Samples were sonicated and incubated in RIPA lysis buffer supplemented with protease inhibitor cocktail and phosphatase inhibitor. Equal amounts of protein, determined by Bradford assay, were resolved by SDS-PAGE and transferred
Membranes were blocked and proteins were detected with primary antibodies. Membranes were then probed with LI-COR IRDye secondary antibodies, which were detected using an Odyssey infrared imaging system (IRDye 800CW Donkey anti-Rabbit (926-32213; 1:10,000) and IRDye 680RD Donkey anti-Goat (925-68074; 1:10,000; LI-COR Biosciences, Lincoln, NE, USA). The following primary antibodies were used in this study: anti-tPA (ASMTPA-GH; 1:1000) and anti-uPA (ASMUPA-GF-HT; 1:1000) from Molecular Innovations (Novi, MI, USA), anti-BDNF (SC-546; 1:200) and anti-actin (SC-1616; 1:2000) from Santa Cruz (Dallas, TX, USA). Band density was determined using Image Studio software. Results were normalized to actin as a load control, and then reported as a ratio to ZT6 (uPA and tPA) or to WT (mBDNF). Positive controls: purified uPA (MUPA) and tPA (MTPA) from Molecular Innovations (Novi, MI, USA). A minimum of 4 biological replicates were run for each experimental condition.

2.3.5 Casein-plasminogen gel zymography

Enzyme activities of uPA and tPA in SCN tissue were examined by casein-plasminogen gel zymography, which provides a preliminary measure of proteolytic activity in the slice. Slices were collected at ZT6, ZT12, ZT16, and ZT23 using the same protocol as for western blots. SCN from 2 mice were pooled for each sample. Slices collected at ZT12 on day 1 in vitro were made at ZT6 (time in vitro = 6 h) and slices collected at ZT12 on day 2 in vitro were made at ZT6 (time in vitro = 30 h). Slices were homogenized and incubated in zymography lysis buffer without protease or phosphatase inhibitors. Equal amounts of protein (by Bradford assay) were resolved by SDS-PAGE using a 10% polyacrylamide gel supplemented with 2 mg/mL casein and 4.5 μg/mL human plasminogen (HGPG; Molecular Innovations). After electrophoresis, gels were incubated in 2.5% Triton-X 100 for 30 minutes twice, incubated in 100 mM Tris buffer, pH 8.8 for 3.5 h at 37 °C, and stained with Coomassie brilliant blue (Sigma-Aldrich). Purified tPA and uPA (Molecular Innovations) were used as positive controls, and proteolytic activity was observed as a clear region of degradation on a dark background. Gels were imaged using an Odyssey infrared imaging system, and band density was determined using Image Studio software. In some cases, gels were imaged using a camera system and band density was determined using ImageJ. Results were reported as a ratio to ZT23. A minimum of 3 biological replicates were run for each experimental condition.
2.3.6 Statistical methods

All statistical analysis was performed using SigmaPlot. Individual animals were the experimental units for the behavioral experiments. Tissue from a single animal (or pooled when necessary) were the experimental units for the electrophysiology and immunoblotting experiments. For comparisons of means in samples with normal distributions and homogeneous variances (as indicated by a Levene’s test), an independent-sample t test or ANOVA was used for comparisons between two means or two or more means, respectively. In cases where normality test failed, a Kruskal-Wallis One Way Analysis of Variance on Ranks was used in place of the ANOVA. Significance was considered to be $P < 0.05$.

2.4 Results

2.4.1 $tPA^{-/-}$ mice exhibit normally entrained behavioral activity rhythms, a slight reduction in the rate of entrainment to new LD, normal phase-shifting response to light pulses, and normal free-running periods

Entrainment

$tPA^{+/−}$ mice appear to show a normal behavioral pattern when entrained to a LD cycle, comparable to that observed in WT mice. When the light cycle was inverted, $tPA^{-/-}$ mice took significantly longer (5.67 ± 0.33 days) than WT mice (3.83 ± 0.40 days) to achieve a stable onset of activity at the new time of lights off ($t_{10} = 3.51$, $P = 0.006$). Visual inspection of actograms suggests that the $tPA^{-/-}$ mice also show increased activity during the light phase during the transition period to the new dark onset (Figure 2.1).

Light-induced phase shifts

In this experiment, the light-induced phase response of mice deficient in tPA was evaluated in comparison to wildtype mice. $tPA^{+/−}$ did not exhibit a significantly different light-induced phase response in comparison to WT when given a light pulse at either ZT16 at 300 ($tPA^{+/−} = -2.1 ± 0.39$ and WT = -2.5 ± 0.59, $t_6 = 0.57$, $P = 0.59$; ), 50 ($tPA^{+/−} = -1.9 ±
Figure 2-1. Entrainment in WT vs tPA−/− mice.

Representative actograms for (A) a C57BL/6J mouse (WT) and (B) a tPA−/− mouse after a 12-h shift in the LD cycle, showing an apparent difference in the time to entrain. Gray denotes dark period (C) Mean time to entrain to a 12-h shift in the light-dark cycle. P < 0.05. n = 6 for each group.
0.08 and WT = -1.8 ± 0.27, \( t_9 = 0.21, P = 0.84 \), or 5 lux (tPA\(^{-/}\) = -1.4 ± 0.19 and WT = -1.2 ± 0.27, \( t_{10} = 0.61, P = 0.55 \)) or ZT22 at 300 lux (tPA\(^{-/}\) = 0.4 ± 0.02 and WT = 0.5 ± 0.16, \( t_3 = 0.47, P = 0.67 \)) (Figure 2.2).

**Free running periods**

The free running period was assessed for 6 WT and 6 tPA\(^{-/}\) mice in DD, then LL, then a second time in DD. There were no significant differences in free-running period between genotypes in the first DD period (WT: 23.78 ± 0.07 h; KO: 23.80 ± 0.07 h, \( t_{10} = 0.17, P = 0.87 \)), LL (WT: 24.81 ± 0.17; KO: 24.85 ± 0.08, \( t_{10} = 0.22, P = 0.83 \)), or the second DD period (WT: 23.84 ± 0.05; KO: 23.79 ± 0.09, \( t_{10} = 0.52, P = 0.61 \)).

### 2.4.2 SCN slices from tPA\(^{-/}\) mice exhibit entrained neuronal activity rhythms that phase-shift in response to glutamate

To further investigate circadian entrainment and phase shifting in tPA\(^{-/}\) mice, we recorded neuronal activity from SCN brain slices of tPA\(^{-/}\) mice in control (untreated) conditions and following glutamate treatment. In tPA\(^{-/}\) brain slices, SCN neuronal activity recorded on day 2 in vitro exhibited a circadian rhythm with a peak during mid-day (Fig 3a). The mean (± SEM) time of peak neuronal activity in control tPA\(^{-/}\) brain slices was at ZT6.1 ± 0.5 (n = 4) (Figure 2.3a). This time of peak is consistent with that of WT mice (ZT6), indicating that the circadian clock of tPA\(^{-/}\) mice exhibits normally entrained neuronal activity rhythms. These results are also consistent with the normal entrainment of behavioral rhythms seen in vivo. Glutamate (1 mM) applied to SCN slices in the early subjective night (ZT16) for 10 minutes delayed the time of peak of neuronal activity to ~ZT9 (Fig 3b), with a mean phase-shift of -3.1 ± 0.7 hr (n = 4). Glutamate (1 mM) applied to the SCN in the late subjective night (ZT23) advanced the time of peak neuronal activity to ~ZT4 (Figure 2.3c). No significant differences were found between WT and tPA\(^{-/}\) at either ZT16 or ZT23. These data are summarized in Figure 2.3d. Together, they indicate that SCN of tPA\(^{-/}\) mice to phase shift in response to glutamate.

To more closely examine potential differences between WT and tPA\(^{-/}\), we generated dose response curves for glutamate-induced phase shifts in SCN brain slices from WT and tPA\(^{-/}\) mice. For these experiments, WT and tPA\(^{-/}\) brain slices were treated at ZT16 with varying concentrations of glutamate for 10 minutes. Glutamate induced
Figure 2-2. Light-induced phase shifts in WT vs tPA^{-/-} mice

Mean phase shift of behavioral activity rhythms of WT and tPA^{-/-} mice in response to a 15-min light pulse at CT 16 (first three bars) or CT 22 (rightmost pair of bars). n = 3–6 per group. X-axis denotes light intensity (lux). No significant differences between pairs at all intensities.
SCN from tPA<sup>−/−</sup> mice exhibit entrained neuronal activity rhythms that phase shift in response to glutamate. Shown here are the 2-h means ± SEM of the spontaneous neuronal activity recorded in single experiments. (A) Control experiment shows peak activity at ZT6. (B) Glutamate (1 mm) treatment at ZT16 induces a ~4-h phase delay. (C) Glutamate (1 mm) at ZT23 induces a ~2-h phase advance. Glutamate-induced phase shifting tPA<sup>−/−</sup> SCN is comparable to shifting in WT. (D) Shown are mean ± SEM phase shifts induced by 1 mm glutamate. Delays are plotted as negative values. In both WT and tPA<sup>−/−</sup> mice glutamate at ZT16 induces an approximately 3-h phase delay, while glutamate at ZT23 induces a phase advance. (E) Varying concentrations of glutamate were applied to WT or tPA<sup>−/−</sup> SCN slices at ZT16 to generate dose–response curves. No significant differences were found when comparing WT to tPA<sup>−/−</sup>, n = 3–4. ZT, Zeitgeber time.
similar phase shifts in tPA\(^+\) and WT brain slices at all the concentrations used (Figure 2.3e). No significant differences were found in the magnitude of phase shifts in the WT and tPA\(^+\) tissue at all concentrations, although there appears to be a trending difference at intermediate glutamate concentrations: 1 µM (tPA\(^+\) = -0.33 ± 0.17 and WT = 0.33 ± 0.44, \(t_4 = 1.41, P = 0.23\)), 10 µM (tPA\(^+\) = -1.83 ± 0.73 and WT = -2.42 ± 0.3, \(t_4 = -0.74, P = 0.5\)), 100 µM (tPA\(^+\) = -1.00 ± 0.58 and WT = -2.08 ± 0.17, \(t_4 = -1.86, P = 0.14\)), or 1 mM (tPA\(^+\) = -3.31 ± 0.47 and WT = -3.08 ± 0.51, \(t_5 = 0.33, P = 0.76\)). Collectively, these results indicate that tPA\(^+\) circadian clock phase shifts in response to \textit{in vitro} glutamate in a manner that does not differ substantially from WT. Again, these results are consistent with our \textit{in vivo} data showing normal photic phase shifting in tPA\(^+\) mice. While on the surface these data seem at odds with our previous work showing that inhibiting tPA prevents glutamate induced phase shifting, alternatively they suggest a potential redundant or compensatory mechanism allowing phase shifting in the circadian clock of tPA\(^+\) mice (Mou \textit{et al.}, 2009b). Given that PAI-1 also inhibits the enzymatic activity of uPA, uPA was a logical alternative mechanism acting in the absence of tPA.

2.4.3 \textit{tPA and uPA are both expressed in the SCN}

We have previously demonstrated that tPA is expressed in the SCN (Mou \textit{et al.}, 2009b). Here we investigated tPA protein expression in SCN brain slices from WT and tPA\(^+\) mice across circadian time. Slices were collected at ZT6, ZT16, and ZT23 on the first day \textit{in vitro} and at ZT6 on the second day \textit{in vitro}, then subjected to western blotting. As shown in Fig 4, in WT SCN we find that tPA expression is higher at night (ZT16 and ZT23) than during the day (ZT6), and this rhythm persists into the second day \textit{in vitro} (one-way ANOVA: \(n = 5, F_6 = 5.56, P = 0.008\)). As expected, no tPA protein was present in tPA\(^+\) SCN tissue.

Next, we used western blotting to assess uPA expression in the SCN of WT and tPA\(^+\) mice across the same time-points used above. Anti-uPA antibody identified an approximately 55 kDa band in SCN samples from WT and tPA\(^+\) brain slices, supporting uPA protein expression in SCN. In both WT and tPA\(^+\) SCN uPA expression was constant, with no significant differences across any groups (one way ANOVA: \(n = 7, H_7 = 3.99, P = 0.78\)), indicating no circadian rhythm in uPA protein expression (Figure 2.4). In addition,
tPA expression is circadian \textit{in vitro}, and uPA expression is constant. Proteins extracted from the SCN were subjected to western blotting. (A) Representative images showing tPA band at ~70 kDa, and actin load control (~42 kDa). tPA bands are not present in the tPA$^{-/-}$.

(B) Histogram showing tPA relative to actin, normalized to ZT6 in WT SCN. One-way ANOVA $P = 0.008$, $n = 5$.

(C) Representative images showing uPA band at ~55 kDa, and actin load control (~42 kDa).

(D) Histogram showing uPA relative to actin, normalized to ZT6 in WT and tPA$^{-/-}$ SCN. $n = 5$–$7$, no significant differences.
there appears to be no compensatory changes in uPA protein expression in SCN tissue from tPA$^{-/-}$ mice.

### 2.4.4 PAI-1 blocks phase delays in tPA$^{-/-}$ mice

tPA and uPA have overlapping functions as plasminogen activators, and while the kinetics of uPA release in the synapse are different than those of tPA, examples of functional redundancy between these proteases have been seen in previous studies (Carmeliet et al., 1994; Leonardsson et al., 1995; Bugge et al., 1996). Therefore, we investigated the possibility that uPA can compensate for the lack of tPA to enable phase shifting in the tPA$^{-/-}$ mice. First, we used PAI-1 to inhibit uPA in SCN brain slices from tPA$^{-/-}$ mice. PAI-1 inhibits both tPA and uPA and blocks phase shifts in WT SCN brain slices with an ED$_{50}$ of 0.6 nM, which is comparable to its concentration in serum (Mou et al., 2009b). Pretreating SCN slices from tPA$^{-/-}$ mice for 30 minutes prior to glutamate treatment with 5 nM PAI-1 blocked glutamate-induced phase delays (mean phase-shift = -0.8 ± 0.4 h, $n = 3$; Glutamate vs Glutamate + PAI-1: $t_5 = -3.38, P = 0.02$) (Figure 2.5). When applied alone for 40 minutes, 5 nM PAI-1 had no effect on the phase of the neuronal activity rhythm. Thus, inhibiting uPA in tPA$^{-/-}$ brain slices prevents glutamate-induced phase delays in neuronal activity rhythms. Since PAI-1 has no known high-affinity targets other than tPA and uPA, this supports the hypothesis that uPA may be compensating for the loss of tPA in the tPA$^{-/-}$ mice.

### 2.4.5 Selective inhibition of uPA prevents glutamate-induced phase resetting in the SCN of tPA$^{-/-}$ but not WT mice

To directly address the involvement of uPA in circadian clock phase shifting, we utilized the uPA specific small molecule inhibitor UK122, which is selective for uPA at low concentrations (uPA ED$_{50}$ = 200nM) (Zhu et al., 2007). SCN slices from WT and tPA$^{-/-}$ mice were treated with 200 nM UK122 for 30 minutes prior to glutamate application at ZT16 and ZT23, and then recordings of neuronal activity rhythms were conducted during the next day. Treating SCN slices from WT mice with 1 mM glutamate + 200 nM UK122 at ZT16 results in a phase delay of 2.8 hr, with a mean time of peak occurring at ZT8.8 ± 0.6 ($n = 3$, Figure 2.6a). 200 nM UK122 applied alone for 40 minutes at ZT16 had no effect (Figure 2.6). These results show that in WT SCN tissue, where tPA is present,
5 nm PAI-1 prevents glutamate-induced phase delays in tPA−/− mice. Shown are mean ± SEM phase-shifts induced by treatments indicated applied to SCN slices of tPA−/− mice. Phase delays plotted as negative values. *P < 0.05, n = 3.
Figure 2-6. Selective inhibition of uPA prevents glutamate-induced phase resetting in the SCN of tPA<sup>−/−</sup> but not WT mice.

Selective inhibition of uPA prevents glutamate-induced phase shifts in tPA<sup>−/−</sup> but not WT mice. Shown are representative trace of SUA recordings from a single SCNs treated with 1 mm glutamate and 200 nm uPA inhibitor (UK122): (A) WT treated at ZT16 shows an ~2.5 h phase delay; (B) tPA<sup>−/−</sup> treated at ZT16 shows peak activity at ~ZT6.57; (C) Shown are mean ± SEM phase shift induced by treatments indicated at ZT16. *P = 0.02 n = 3; (D) WT treated at ZT23 shows an ~3 h phase advance; (E) tPA<sup>−/−</sup> treated at ZT23 shows peak activity at ~ZT6.75; and (F) Mean ± SEM phase shift induced by treatments indicated at ZT23. *P = 0.02, n = 3.
inhibiting uPA is not sufficient to prevent glutamate-induced phase delays of the SCN circadian clock (WT: Glutamate vs Glutamate + UK122 at ZT16: $t_4 = -0.318, P = 0.77$). However, when SCN slices from tPA$^{-/-}$ mice were treated at ZT16 with glutamate + UK122, the uPA inhibitor prevented glutamate-induced phase delays (mean phase shift = $-0.4 \pm 0.3 \text{ h}, n = 3$, Figure 2.6b) (tPA$^{-/-}$: Glutamate vs Glutamate + UK122 at ZT16: $t_5 = -3.35, P = 0.02$). Again, application of UK122 alone for 40 minutes at ZT16 had no effect on the phase of SCN neuronal activity rhythm (Figure 2.6). At ZT23, treating WT slices with 1 mM glutamate + 200 nM UK122 resulted in a phase advance of $3.34 \pm 0.35 \text{ h}$, while the same treatment in tPA$^{-/-}$ SCN slices resulted in no phase shift, with a mean time of peak occurring at $ZT6 \pm 0.58 \text{ h}$ (Figure 2.6f). Thus, inhibiting uPA prevents glutamate-induced phase advances in tPA$^{-/-}$ SCN but not WT SCN (WT: Glutamate vs Glutamate + UK122 at ZT23: $t_4 = -1.80, P = 0.15$; tPA$^{-/-}$: Glutamate vs Glutamate + UK122 at ZT23: $t_4 = 3.51, P = 0.03$.) Collectively, this suggests that uPA contributes to circadian clock phase shifting in tPA$^{-/-}$ mice, further confirming uPA as a mechanism of redundancy within the SCN.

### 2.4.6 tPA and uPA enzymatic activity in the SCN

In addition to assessing their overall expression, it is important to determine the proteolytic activity of these proteases. We used casein-plasminogen gel zymography to assess tPA and uPA proteolytic activity in the SCN. WT and tPA$^{-/-}$ SCN slices were collected at ZT6, ZT12, ZT16, and ZT23 on the first day in vitro, and at ZT6 and ZT12 on the second day in vitro. Equal amounts of total protein extracted from the SCN in the absence of protease inhibitors were subjected to casein-plasminogen gel zymography. Two regions of degradation were present in WT SCN: one band at $\approx 70 \text{ kDa}$ corresponding with the tPA positive control and one band at $\approx 33 \text{ kDa}$ corresponding with the uPA positive control. These results confirmed the presence of proteolytically active uPA and tPA in the SCN of WT mice.

We found no significant differences in tPA proteolytic activity in WT SCN across all time points (one way ANOVA: $n = 3$ to 15, $H_5 = 4.63, P = 0.46$; Figure 2.7a-c). As expected, no tPA proteolytic activity was present in the tPA$^{-/-}$ SCN tissue. uPA proteolytic activity in both WT and tPA$^{-/-}$ SCN increased over the first $\approx 24 \text{ h}$ that slices were maintained in vitro, and then appeared to stabilize at this higher level, but it did not exhibit a circadian rhythm (one-way ANOVA: $n= 3$ to 15, $H_5 = 40.91, P < 0.001$; Fig 7d). These
tPA proteolytic activity is not circadian in the mouse SCN in vitro. (A) Representative casein-plasminogen gel zymograms of protein extracts from SCN of WT and tPA−/− mice collected at ZT6, ZT16, ZT23 and a second day in vitro ZT6 and (B) an extended time course of protein extracts from WT SCN collected at ZT12, ZT23, and second day in vitro ZT6 and ZT12 (all samples are presented in chronological order). tPA (~70 kDa) and uPA (~30 kDa) proteolytic activity appears as clear bands of degradation on a dark background that are distinguishable based on molecular weight. (C) Shown is tPA proteolytic activity in WT SCN normalized to ZT23, no significant differences in One-way ANOVA, n = 3–15. tPA proteolytic activity is not present in the tPA−/− SCN. (D) Shown is mean ± SEM relative uPA proteolytic activity in WT SCN normalized to ZT23, One-way ANOVA P < 0.001, n = 3–15. Increase is correlated with increasing time in vitro, not ZT. (E) uPA proteolytic activity is not significantly different in tPA−/− mice when compared to WT normalized to ZT6. Shown are means ± SEM relative uPA activity from WT and tPA−/− SCN collected at the indicated time points. No significant differences when comparing WT and tPA−/− at each ZT, n = 7.

Figure 2-7. tPA and uPA activity in WT and tPA−/− SCN.
results suggest that neither tPA nor uPA total proteolytic activity in the SCN are under circadian control in vitro.

When comparing tissue from tPA−/− and WT mice there were no significant differences in uPA activity at any time-point (Figure 2.7e). This indicates that in tPA−/− SCN tissue uPA’s involvement in glutamate-induced phase shifting does not involve a compensatory change in proteolytic activity. Rather, the data support the hypothesis that uPA and tPA exhibit redundancy in function.

2.4.7 2.4.g BDNF protein expression

To assess the involvement of BDNF in our experiments, SCN brain slices from both WT and tPA−/− mice received no treatment or were treated with 200 nM UK122 at ZT16 and relative amounts of mBDNF were assessed via western blotting. We found that mBDNF levels relative to actin were significantly lower in tPA−/− SCN tissue vs. WT SCN tissue (t14 = 3.38, P = 0.004). Treatment with UK122 did not induce significant changes in either WT or tPA−/− tissue (Figure 2.8).

2.5 Discussion

Previous research from our lab supports tPA regulation of circadian clock phase resetting (Mou et al., 2009b). PAI-1 blocks glutamate-induced phase shifts of the SCN circadian clock in tissue from WT mice but not from mice lacking the PAI-1 stabilizing protein, vitronectin (Mou et al., 2009b). Adding plasmin or mBDNF, but not plasminogen or proBDNF, recovers glutamate-induced phase shifting. Furthermore, mBDNF protein expression levels are higher in the SCN at night than during the daytime (Liang et al., 1998). Together, these data support a model where tPA levels increase at night, allowing activation of plasminogen into plasmin to cleave proBDNF into mBDNF. mBDNF activation of TrkB receptors concurrent with glutamate activation of NMDAR shifts SCN circadian clock phase (Liang et al., 1998; Liang et al., 2000; Michel et al., 2006; Mou et al., 2009b).

Here, we expanded our investigation of the plasminogen activating system in the SCN by assessing circadian function in tPA−/− mice. Somewhat surprisingly, these mice exhibit normal entrainment to 12:12 LD cycles. However, when the light-dark cycle is shifted by 12 hours we observe a significant increase in the amount of time needed to entrain to the new cycle. This could indicate a deficiency in the ability of these mice to shift
mBDNF levels are lower in tPA−/− SCN tissue, but are not affected by UK122 (200 nm). (A) Shown are representative blots of mBDNF (~17 kDa) and actin load control (~42 kDa) from WT and tPA−/− SCN which were treated as indicated. (B) Histogram showing mean ± SEM mBDNF relative to actin, normalized to the first WT sample on each blot. A significant difference was found when comparing untreated WT to tPA−/−. One-way ANOVA: n = 4–10; P < 0.05.

Figure 2-8. BDNF protein expression.
their clock, which would be consistent with the trend towards smaller glutamate-induced phase shifts observed in vitro. An alternative possibility is that the slower reentrainment is related to a decrease in photic masking as increased locomotor activity may reduce the phase shifting effects of light. It should be noted that because of the potential for photic masking, we cannot state for certain when an animal is entrained – we can say that the animal appears to be entrained based on the behavioral output. However, the rate of apparent reentrainment seen here in WT mice are not inconsistent with other experiments using large changes in the LD cycle. Hannibal et al (2008) reported reentrainment to an 8-hour shift in two days, and Sellix et al (2012) showed 4 days to reentrain to a 6 hour shift. Time to reentrain may depend on the environment in which the animals were raised, light intensity and/or spectrum, and other factors that differ between labs.

Additional experiments demonstrated that the tPA−/− mice exhibit no differences from WT mice with respect to their phase shifting responses to light pulses presented at CT16 and CT-22, over a wide range of intensities. Moreover, the tPA−/− mice do not differ from WT mice in their free running periods in DD or LL. Thus, our initial behavioral assessments of these mice indicate a deficiency when exposed to a dramatic inversion of LD cycles, but no overt differences from WT in photic responsiveness to light pulses.

We also find no severe deficiencies in our in vitro assessment of SCN neuronal activity in tPA−/− brain slices. The circadian rhythms in SCN spontaneous neuronal activity are not different from WT SCN, indicating the mice entrain to the 12:12 LD cycle. These rhythms phase shift in response to glutamate, undergoing phase delays in time-of-peak neuronal activity when glutamate is applied at ZT16 and phase advances when glutamate is applied at ZT23. Moreover, glutamate-induced phase shifting in tPA−/− brain slices does not differ significantly from WT brain slices with respect to magnitude of shift or in its dose dependency. Given PAI-1’s clear inhibition of glutamate-induced phase shifts in vitro, these results suggest differences between the effects of pharmacological inhibition and genetic reduction of tPA (Mou et al., 2009b). Since PAI-1 inhibits both tPA and uPA activity, we shifted our attention to uPA.

uPA is an ideal candidate for functional redundancy/compensation for tPA in the SCN because it also cleaves plasminogen to form plasmin. Consistent with this hypothesis, we found that uPA is expressed in the SCN. We assessed potential circadian rhythms in tPA and uPA expression, and found that tPA protein levels are higher in the
night than during the day in the SCN. Conversely, there is no apparent circadian rhythm in uPA protein expression in WT or tPA−/− SCN tissue. Additionally, uPA protein levels are not different between WT and tPA−/− SCN tissue, suggesting that there is no compensatory effect on uPA protein expression in the developmental absence of tPA. These findings support the idea that uPA may play a role in phase shifting, but at the same time the unique expression patterns of tPA and uPA point to potential differences in their regulatory and functional mechanisms. Importantly, our assessments of tPA and uPA expression do not account for potential differences in their release or cell-type specific localization. Experiments utilizing in cell culture methods and immunohistochemistry will help clarify potential differences in these parameters.

In assessing uPA function in the SCN, we find that PAI-1 inhibits glutamate-induced phase delays in tPA−/− mice brain slices. Moreover, the uPA-specific inhibitor UK122 does not prevent glutamate-induced phase delays or advances of neuronal activity rhythms in SCN from WT mice, but it does inhibit these phase shifts in SCN from tPA−/− mice. These results are important for two reasons. First, they strengthen the conclusion that uPA can support glutamate-induced phase shifts in the absence of tPA. Second, they indicate a dynamic interplay between tPA and uPA, suggesting a system where neither protease is necessary, but each sufficient to enable phase shifting. Importantly, it appears that at least one plasminogen activator is required for phase shifting to occur. Experiments in uPA knockout mice will be important to further investigate the roles of these proteases in circadian clock phase shifting.

We also investigated tPA and uPA enzymatic activity in the SCN. tPA proteolytic activity does not exhibit a circadian rhythm despite finding higher protein levels at night. uPA proteolytic activity also does not show a circadian rhythm in the SCN. However, uPA proteolytic activity increases in parallel with the duration of time the brain slices are maintained in vitro, despite finding constant protein levels. uPA is secreted as a 55 kDa high molecular weight (HMW) pro-enzyme that is cleaved into a 2-chain HMW enzyme that is most active when bound to the uPA receptor (Husain, 1991; Ronne et al., 1991). The 2-chain HMW uPA can be further cleaved into 2 individual chains: a ~33 kDa active low molecular weight (LMW) uPA and a ~22 kDa non-active amino terminal fragment (Lijnen et al., 1987a; Novokhatny et al., 1992). Because of the different extraction procedures, our western blotting results assessed the uncleaved ~55 kDa HMW uPA,
while our zymography results address the ~33 kDa active LMW uPA. Thus, our data could support distinct regulation of the two forms of uPA in the SCN, a possibility that requires further exploration. There are reports of uPA involvement in neuronal repair processes, so it is possible that the increased uPA proteolytic activity we observe across time in vitro is in response to the tissue injury (Lahtinen et al., 2006; Lahtinen et al., 2010; Lukasiuk et al., 2011; Cho et al., 2012; Karagyaur et al., 2015). Collectively, these results suggest that uPA and tPA proteolytic activity are regulated by distinct mechanisms in the SCN. It is also noteworthy that these proteins are regulated through multiple protein interactions in the intact system, and these interactions would be disrupted during the tissue extraction procedures used here (Collen & Lijnen, 2005; Melchor & Strickland, 2005). Therefore, it is possible that the activity patterns we see in vitro differ from in vivo. Studies utilizing in situ zymography and cell culture procedures will enhance characterization of the proteases in the SCN.

Previous studies investigating tPA and uPA redundancy indicate that uPA can compensate for the absence of tPA through a mechanism that is not related to increases in protein expression (Siconolfi & Seeds, 2001; Bukhari et al., 2011). Studies assessing tPA and uPA as mediators of psychostimulant-induced synaptic plasticity and remodeling demonstrated divergent roles for these plasminogen activators (Bahi & Dreyer, 2008; Al Maamari et al., 2014). In the SCN, we find no statistically significant differences in either uPA expression or uPA proteolytic activity between WT and tPA−/− tissue. While noting the same caveats with respect to in situ regulation, these results suggest that uPA’s involvement in SCN clock phase shifting in tPA−/− mice is not related to compensatory differences in either the expression or activity of uPA. However, to verify that there is no cross-compensation between these two proteases, it will be important to assess tPA expression and activity in the SCN of uPA−/− mice.

Both tPA and uPA generate plasmin, which can cleave proBDNF into mBDNF. BDNF regulates NMDA currents in the SCN (Kim et al., 2006; Michel et al., 2006), and therefore mBDNF generation downstream of the plasminogen activators may contribute to SCN clock phase shifting. Distinctions between the two proteases in the degree to which they regulate mBDNF production are unclear: there are numerous studies linking tPA directly to BDNF maturation, but many fewer demonstrating uPA involvement (Pang & Lu, 2004; Pang et al., 2004; Gray & Ellis, 2008; Cunha et al., 2010; Rodier et al., 2014).
Our results add to this literature by demonstrating, first, that mBDNF levels are decreased by about 50% in tPA−/− SCN tissue relative to WT levels. This is consistent with the effects seen in other brain regions (e.g., Pang et al., 2004). This suggests that tPA participates in regulating BDNF maturation in the SCN, but that other proteases are able to function in this manner as well. Secondly, the data suggest that uPA does not contribute to BDNF cleavage in the SCN. Thus, the mechanism(s) through which uPA modulates glutamate-induced phase resetting in the SCN appear to be distinct from those through which tPA acts.

As an additional layer of complexity, BDNF stimulates tPA expression (Fiumelli et al. 1999), enhances uPA release from microglia (Nakajima 1998, 2005), and increases uPA production (Sun et al. 2006). Whether BDNF modulates tPA and/or uPA expression in the SCN is an interesting question that remains to be determined.

Although our data show similarities between the actions of tPA and uPA, this does not abrogate them each having actions in the SCN not shared by the other, a conclusion supported by our mBDNF data; while their proteolytic activities overlap, the two proteins also display considerable differences. tPA’s interactions with several membrane-bound receptors, including NMDAR, annexin II, LRP-1, and epidermal growth factor receptor (EGFR), can influence neuronal responses (Melchor & Strickland, 2005). Conversely, uPA binding to uPA receptor (uPAR) regulates uPA dependent proteolysis, cell adhesion, and signaling (Smith and Marshall 2010). Therefore, tPA’s various receptor interactions and uPA’s uPAR-dependent processes represent unexplored avenues that could influence SCN circadian clock phase regulation. Additionally, shared functions of tPA and uPA in the SCN could include processes down-stream of plasmin activation that don’t involve BDNF. Plasmin cleaves the extracellular matrix (ECM) to allow cell structure reorganization, which could be relevant to clock function as several studies have demonstrated circadian rhythms in micro-cellular rearrangements in the SCN (Soleman et al., 2013). Plasmin regulation of matrix metalloproteinase (MMP) activity could also influence circadian clock function (Kaur et al., 2004). MMP’s could provide an additional mechanism for ECM rearrangement, may be able to feed back into mBDNF generation, and can influence neuronal responses via modulation of NMDAR activity.

Taken together, these data support a more complex scenario of proteolytic involvement in SCN circadian clock phase regulation than originally described. In addition
to tPA activating plasmin to generate mBDNF, we now have evidence that uPA can support glutamate induced phase resetting, and that uPA acts through distinct mechanism(s). Given the paucity of information on uPA’s actions in regulating synaptic plasticity together with the known differences in their signaling mechanisms, these results create a wonderful opportunity to explore the shared vs. distinct actions of these proteases in a well-defined model system of synaptic plasticity – the SCN circadian clock. Moreover, further clarifying how these plasminogen activators function in the SCN to modulate photic signaling will expand our understanding of circadian clock phase regulation.

In conclusion, our data reinforce the concept that extracellular proteases are involved in SCN circadian clock phase regulation. By demonstrating a critical role for uPA and tPA, these results add substantially to those from our previous study with respect to our knowledge of plasminogen activators modulating plasticity within the SCN. The possibility that these two proteases may serve functionally redundant roles in the SCN, and that neither is itself necessary for normal circadian function, would not be unprecedented. Regarding the SCN circadian clock, functional redundancy appears to be the norm and these data reinforce this concept (Hastings 2014). Our in vivo studies on tPA−/− mice tell a similar story, with the genetic deletion of tPA causing minimal disruption in behavioral entrainment or light-induced phase shifting, although these mice take longer to entrain to a 12 h shift in the LD cycle. A full understanding of plasminogen activator functions in SCN clock regulation, including potential interactions with uPAR and/or LRP-1 receptors, and extracellular matrix proteins is clearly warranted. Continued exploration of extracellular protease activities in the SCN should provide greater insight into the cellular processes modulating circadian clock functioning.
3  LOW DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN-1 REGULATES GLUTAMATE-INDUCED PHASE SHIFTING IN THE MOUSE SUPRACHIASMATIC NUCLEUS
3.1 Abstract

Glutamate induces phase shifts of the mammalian suprachiasmatic nucleus (SCN) circadian pacemaker by activating NMDA receptors (NMDARs). These phase shifts are restricted to the subjective night by rhythmic activities of mature brain derived neurotrophic factor (BDNF), TrkB receptors, NMDARs and the plasminogen activators (PAs), t(issue-type)PA and u(rokinese)PA. These phase shift gating proteins are all known to interact with low-density lipoprotein receptor-related protein 1 (LRP-1), and here we investigate whether LRP-1 is also a regulator of SCN clock function. We investigated LRP-1 expression in the SCN via western blotting, and find that LRP-1 is expressed in the SCN and that it exhibits both diurnal and time-in-dish dependent variations in expression patterns. Using electrophysiological recordings of SCN neuronal activity rhythms, we find that inhibiting LRP-1 with receptor associated protein (RAP) or an LRP-1 specific antibody in SCN brain slices prepared from wild-type (WT; C57BL/6) mice prevents glutamate-induced phase delays and advances in neuronal activity rhythms at ZT16 and ZT23, respectively. RAP also prevents glutamate-induced phase delays in tPA knockout mouse (tPA−/−; B6.129S2-Plattm1Mlg/J) SCN slices. Treating WT SCN slices with combinations of glutamate and RAP does not alter BDNF maturation or TrkB receptor phosphorylation on Y680/681. Treating SCN brain slices with RAP reduces NMDAR NR2B subunit phosphorylation on S1480, but does not change phosphorylation on Y1472. Collectively, these results indicate that LRP-1 participates in SCN circadian clock phase regulation, and that it acts through mechanism(s) that do not require plasminogen activator activity, but may involve changes in NMDAR localization.

3.2 Introduction

In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus serves as the primary circadian pacemaker (Moore & Eichler, 1972; Stephan & Zucker, 1972). It orchestrates daily rhythms in behavior in physiology by generating endogenous 24 h cycles, synchronizing them to the environment, and distributing the timing cues to the body. The primary synchronizing signal in the environment is light, which modulates SCN clock phase through a process known as photic entrainment. Photic entrainment relies on light stimulating melanopsin-expressing retinal ganglion cells to release glutamate onto
SCN neurons (Liou et al., 1986; Ding et al., 1994; Porterfield et al., 2007; Porterfield & Mintz, 2009). Glutamate binds to and activates NMDA receptors (NMDAR), inducing a calcium influx that stimulates downstream signaling events, ultimately leading to shifts in the timing of the circadian clock (Ding et al., 1997; Gillette & Tischkau, 1999; Butcher et al., 2003; Pizzio et al., 2003; Butcher et al., 2004; Marpegan et al., 2004; Butcher et al., 2005). The downstream signaling events mediating these phase shifts involve activation of CaMKII by autophosphorylation of Thr286/287 (Golombek & Ralph, 1995; Fukushima et al., 1997; Yokota et al., 2001), which in turn activates neuronal nitric oxide synthase (nNOS) to produce nitric oxide (NO) (Ding et al., 1994; Ding et al., 1997; Melo et al., 1997; Agostino et al., 2004). Through distinct intracellular pathways (Weber et al., 1995; Ding et al., 1998; Prosser, 1998b; Tischkau et al., 2003), these initial signaling events lead to changes in the transcription of core clock genes that can alter the phase of the clock (Ginty et al., 1993; Ding et al., 1997; Gau et al., 2002) in a time-dependent manner: glutamate induces phase delays in the early night, phase advances in the late night, and has no effect on clock phase in the daytime. The mechanisms regulating the differential responsiveness have not been fully elucidated (Iyer et al., 2014).

LRP-1 is a large membrane bound endocytic and signaling receptor that modulates neuronal activity throughout the central nervous system (CNS) (Lillis et al., 2008), and whose function overlaps with processes known to gate phase shifting of the SCN circadian clock. LRP-1 is a member of the low-density lipoprotein (LDL) receptor family that binds over 50 different ligands extracellularly, and upon ligand binding it mediates rapid endocytosis and/or activation of signal transduction cascades (Lillis et al., 2008). LRP-1 is composed of a 515 kDa extracellular α subunit that is non-covalently associated with an 85 kDa transmembrane β subunit (Kerrisk et al., 2014; Ramanathan et al., 2015). The αLRP-1 subunit contains cysteine-rich complement-type ligand binding repeats, and EGF repeats and β-propeller domains that function in the release of ligands (Lillis et al., 2005). The βLRP-1 subunit contains a single transmembrane domain and cytoplasmic domain, which has two NPxY motifs that can be phosphorylated to contribute to endocytosis and signal transduction (Lillis et al., 2005). LRP-1 is widely expressed throughout the CNS, and while deletion of the LRP-1 gene is embryonically lethal, neuronal specific deletion of LRP-1 in mice results in severe behavioral and motor abnormalities, including hyperactivity, tremor, and dystonia (May et al., 2004; Liu et al., 2010). LRP-1 can be
expressed in the post-synapse, where it regulates synaptic structural and functional integrity, partly through regulating glutamate receptors (May et al., 2004; Liu et al., 2010).

One prominent group of LRP-1 ligands, the plasminogen activators (tissue type plasminogen activator; tPA and urokinase type plasminogen activator; uPA), act to gate glutamate-induced phase shifts in the SCN (Mou et al., 2009b; Cooper et al., 2017; Cooper, Submitted). tPA is a secreted serine protease that is widely expressed in the brain and has well-characterized roles as a neuromodulator (Melchor & Strickland, 2005; Benarroch, 2007; Medcalf, 2017). uPA is also expressed in the CNS, and while less is known about its neuromodulatory roles, evidence supports its involvement in neuronal repair processes (Merino et al., 2017b). tPA expression is rhythmic in the SCN, with higher levels at night, and inhibiting tPA and uPA with plasminogen activator inhibitor-1 (PAI-1) prevents in vitro glutamate-induced phase shifts of the mouse SCN circadian clock (Mou et al., 2009b; Cooper et al., 2017). tPA facilitates glutamate-induced phase shifts by acting upstream of BDNF, another well-established regulator of glutamate-induced phase shifts (Liang et al., 2000; Pang & Lu, 2004; Pang et al., 2004; Michel et al., 2006; Mou et al., 2009b). tPA cleaves plasminogen into plasmin, which cleaves proBDNF into mBDNF (Pang et al., 2004; Mou et al., 2009b). BDNF levels are also rhythmic in the SCN, such that high night levels of mBDNF bind to TrkB receptors to enable glutamate-induced phase shifts (Allen et al., 2005; Mou et al., 2009b). Surprisingly, tPA knockout mice exhibit no severe deficits in phase-shifting in vivo and in vitro, and uPA appears to compensate, allowing phase shifting in tPA deficient animals (Cooper et al., 2017). However, inhibiting uPA does not influence BDDF maturation in SCN slices, suggesting that this functional compensation may be achieved through BDNF-independent mechanisms (Cooper et al., 2017).

The plasminogen activators also have non-proteolytic functions in the brain, and one major interaction mediating tPA effects is LRP-1 binding. Upon binding, tPA can activate LRP-1 dependent signaling events (Yepes et al., 2003; Zhang et al., 2009b; Mantuano et al., 2013) and LRP-1 endocytic activity can modify tPA proteolytic activity (Casse et al., 2012b). In rat Schwann cell primary cultures, LRP-1 induces c-Jun phosphorylation downstream of tPA binding, which ultimately allows it to function as an injury detection receptor (Flutsch et al., 2016). tPA binding to LRP-1 activates ERK and AKT pathways to promote neurite outgrowth, and tPA induces ERK activation through an
LRP-1 dependent pathway to mediate axonal outgrowth (Fuentealba et al., 2009; Shi et al., 2009; Qian et al., 2016). tPA enhances long term potentiation (LTP) in hippocampal slices by binding LRP-1 and activating cAMP/PKA pathways (Zhuo et al., 2000). In other cases, tPA-dependent effects may involve interactions with other receptors that depend on LRP-1 activity. For example, tPA potentiates NMDAR calcium influx in an LRP-1 dependent manner (Samson et al., 2008). LRP-1 is also required for tPA-mediated microglial activation in the CNS following middle cerebral artery occlusion (MCAO) (Zhang et al., 2009a; Zhang et al., 2009b). In addition to these signaling responses, LRP-1 contributes to tPA recycling in the nervous system. Glutamate induces tPA recycling by astrocytes via LRP-1 dependent endocytosis and subsequent release of tPA (Casse et al., 2012b). LRP-1 may also act as a co-receptor increasing tPA activity (Su et al., 2017). Thus, there is a complex association between tPA and LRP-1 mediated functions in the nervous system.

A second link between LRP-1 and processes gating circadian clock phase shifting involves LRP-1 interactions with NMDA receptors. NMDARs are the primary receptors responsible for photic/glutamate phase shift responses, and there is evidence suggesting rhythms in the NR2B subunit mRNA and protein expression and phosphorylation patterns (Bendova et al., 2012). LRP-1 interacts with NMDAR via PSD95 (May et al., 2004). Inhibiting LRP-1 can decrease NMDAR calcium influx (Bacskai et al., 2000), in part through LRP-1 modulation of NMDAR trafficking and degradation, thus controlling NMDAR surface localization (Maier et al., 2013). This pathway is further influenced by interactions with Trk receptors: LRP-1, NMDAR, and Trk receptors act in complex to mediate tPA dependent calcium influxes through the NMDAR (Martin et al., 2008), and LRP-1 mediates transactivation of Trk receptors by a Src family kinase (SFK) dependent pathway (Shi et al., 2009). LRP-1 may also indirectly influence NMDAR-dependent signaling by regulating the activity of tPA.

Finally, LRP-1 signaling mechanisms also intersect with intracellular signaling events associated with glutamate-induced phase shifts. CaMKII binds LRP-1’s intracellular domain on Y4506, found on an intracellular NPxY motif that is associated with LRP-1 endocytosis and signaling events, preferentially associating with the unphosphorylated form of LRP-1 (Guttman et al., 2009). NMDAR activation of pCREB in primary cortical neuron cell cultures is reduced in LRP-1 deficient neurons (Nakajima et
Finally, as mentioned earlier, LRP-1 rapidly upregulates and activates ERK1/2 (Campana et al., 2006; Mantuano et al., 2008).

Since LRP-1 influences several key players in the SCN circadian clock phase shifting pathway, including the tPA proteolytic cascade, Trk receptor signaling, and NMDAR signals, we hypothesized that LRP-1 contributes to glutamate-induced phase shifting in the mouse SCN in vitro. To address this, we assessed LRP-1 expression in the SCN, whether it affects in vitro glutamate-induced phase shifts, and potential cellular mechanisms through which it may act in the SCN, with a specific focus on interactions with tPA or NMDARs. Our results demonstrate for the first time that LRP-1 is expressed in the SCN and that it modulates glutamate-induced phase resetting.

3.3 Materials and Methods

3.3.1 Animals

All experiments used C57BL/6 wildtype (WT) male mice from Envigo (Indianapolis, IN), or tPA knockout (B6.129S2-Plattm1Mlg/J; tPA−/−) male mice. Male and female tPA−/− mice were generated at and purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). The tPA−/− mice were fully backcrossed for at least 8 generations into the same background C57BL/6 strain as WT, and the C57BL/6 strain is the recommended control for the tPA−/− mice. tPA−/− mice were bred and group-housed in the University of Tennessee Knoxville animal facility in a 12 h-light/12 h-dark cycle (12L:12D) and fed ad libitum. Male mice 6-12 weeks of age at the beginning of each experiment were used, and animals used for each study were age-matched as closely as possible. Experiments were conducted in accordance with the University of Tennessee Knoxville Institutional Animal Care And Use Committee.

3.3.2 Brain slice preparation

Coronal brain slices (500 μm) containing the SCN were prepared from brains dissected following rapid decapitation of unanaesthetized WT or tPA−/− mice. Slices were maintained in a Hatton-style brain slice dish, perfused constantly with oxygenated Earle’s Balanced Salt Solution (EBSS; Sigma-Aldrich, St. Louis, MO, USA) supplemented with glucose, bicarbonate, and gentamicin (pH 7.4) at 37°C, as previously described (Prosser,
2003; Yamada & Prosser, 2014). One mouse was used for each experiment and replicate experiments were performed on different mice.

3.3.3 Slice treatments

Drug treatments were bath applied on the first day in vitro following previously established protocols (Prosser, 2003; Yamada & Prosser, 2014). At zeitgeber time (ZT)16 or ZT23 (ZT0 = lights on in the donor animal colony, and ZT12 = lights off) perfusion was stopped and the medium in the slice chamber was replaced with EBSS supplemented with RAP (50-500 nM, Molecular Innovations Novi, MI, USA), rabbit polyclonal anti-LRP-1 antibody (75 μg/mL, R2629, a generous gift from Dudley Strickland, University of Maryland), and/or glutamate (1 mM; Sigma-Aldrich). For RAP experiments, slices were pretreated with for 5 minutes with RAP only, followed by 10-minute treatment with 1 mM glutamate + RAP, and then 5 minutes post-treatment with RAP only. For R2629 experiments, slices were pretreated with R2629 only for 20 minutes, then treated for 10 minutes with 1 mM glutamate + R2629. In both cases, after the final incubation the medium was replaced with normal EBSS and perfusion was reinstated. Glutamate alone treatments were applied for 10 minutes starting at ZT16 or ZT23. RAP or R2629 only treatments were applied for 20 or 30 minutes, respectively, with a start time corresponding to that of the inhibitor + glutamate experiments.

3.3.4 Single unit activity (SUA) recordings

On the second day in vitro extracellular single unit recordings of neuronal activity were made using methods previously described (Prosser et al., 1994a; Prosser et al., 1994b; Ding et al., 1997; Prosser, 1998a; Soscia & Harrington, 2004; Tischkau et al., 2004). The electrical signal from an individual neuron was isolated using a glass micropipette containing 3M NaCl, and the cell’s activity was recorded for 5 minutes. The electrode was then moved to record the electrical signal from a new cell. Sampling started in the subjective morning (~ZT3) and neuronal activity was sampled for about 10 hr. The firing rates of individual cells were grouped into 2 h running averages, and the time of peak neuronal activity was determined as the time of symmetrically highest activity. Each phase shift was determined by calculating the difference between time-of-peak in untreated
slices vs drug-treated slices. Three to six biological replicates were performed for each experimental condition.

### 3.3.5 Western blots

Brain slices (reduced in size from those for electrophysiology experiments) containing the SCN and the underlying optic chiasm were prepared and maintained as described above. Slices (treated or untreated) were collected at various times and immediately frozen for western blotting experiments. For time-course experiments, untreated slices were collected at ZT6, ZT16, and ZT23. To control the amount of time slices were maintained in vitro, the following slicing and collecting paradigm was used: slices collected at ZT6 on day 1 in vitro were prepared at ZT2 (time in vitro = 4 h); slices collected at ZT16 on day 1 in vitro were prepared at ZT8 (time in vitro = 8 h); slices collected at ZT23 on day 1 in vitro were prepared at ZT3 (time in vitro = 20 h); and slices collected at ZT6 on day 2 in vitro were prepared at ZT10 (time in vitro = 20 h). Bath application drug treatments for western blotting were performed as follows: a) 1 mM glutamate applied for 10 minutes starting at ZT16, then allowed to recover for 5 minutes in normal media before collecting; b) 1 mM glutamate + 100 nM RAP: Slices were treated for 5 minutes with 100 nM RAP, then for 10 minutes with 1 mM glutamate + 100 nM RAP, then 5 minutes 100 nM RAP; c) 100 nM RAP: Slices were treated for 20 minutes with 100 nM RAP then immediately collected; d) untreated controls were collected at ZT16.

Slices were sonicated and incubated in RIPA lysis buffer supplemented with protease inhibitor cocktail (Thermo Scientific, Waltham, MA) and phosphatase inhibitor (Thermo Scientific). Equal amounts of protein, determined by Bradford assay, were resolved by SDS-PAGE (LRP-1 resolved on 3-8% Tris-Acetate gel in non-reducing conditions; non-covalently connected 515 kDa αLRP-1 and 85 kDa βLRP-1 subunits dissociate) and transferred to polyvinylidene fluoride (PVDF) FL immobilon membrane (EMD Millipore, Billerica, MA) using the Transblot turbo system (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked and proteins were detected with primary antibodies. Membranes were then probed with LI-COR IRDye secondary antibodies, which were detected using an Odyssey infrared imaging system (IRDye 800CW Donkey anti-Rabbit (926-32213; 1:10 000) and IRDye 680RD Donkey anti-Goat (925-68074; 1:10 000; LI-COR Biosciences, Lincoln, NE, USA). The following primary antibodies were used...
in this study: anti-actin (SC-1616; 1:2000), anti-phospho-Trk Y680/681 (sc-7996-R, 1:1000, pTrk), anti-phospho-LRP-1 Y4507 (sc-33049, 1:1000, pLRP-1), and anti-BDNF (sc-546, 1:200) from Santa Cruz (Dallas, TX, USA); anti-LRP-1 [5A6] (ab28320, 1:1000, recognizes βLRP-1), anti-CaMKII (ab22609; 1:1000), and anti-phospho-NMDAR2B S1480 (ab73014; 1:1000) from Abcam (Cambridge, MA, USA); anti-phospho-NR2B Y1472 (AB5403; 1:1000) from EMD Millipore (Darmstadt, Germany); anti-phospho-CaMKII Thr286 (p1006-286, 1:1000) from PhosphoSolutions (Auroro, CO, USA) and anti-LRP-1 (R2629; 1:1000, recognizes αLRP-1) a gift from Dudley Strickland (University of Maryland, MD, USA). Band density was determined using Image Studio software. Results were normalized to actin as a load control, then reported as a ratio to ZT6 (time-courses) or to no treated control (treatment experiments). The SCN of a single mouse was used for each sample, and replicate experiments were performed on different mice. Three to nine biological replicates were run for each experimental condition.

### 3.3.6 Statistical methods

All statistical analyses were performed using GraphPad Prism. Tissue from a single animal was the experimental unit for the electrophysiology and immunoblotting experiments. For comparisons of means in samples with normal distributions and homogenous variances (as indicated by a Levene’s test), an independent-sample t test or ANOVA was used for comparisons between two means or more than two means, respectively. Significance was considered to be p < 0.05.

### 3.4 Results

#### 3.4.1 LRP-1 expression and phosphorylation in the SCN

LRP-1 is widely expressed throughout the brain, and here we investigated LRP-1 expression in the mouse SCN in vitro across circadian time. SCN-containing brain slices were collected at ZT6, ZT16, and ZT23 on the first day in vitro and at ZT6 on the second day in vitro, and subjected to western blotting analysis. Anti-LRP-1 antibody (R2629) identified an approximately 515 kDa band, consistent with the αLRP-1 heavy subunit, and the 5A6 anti-LRP-1 antibody detected an approximately 85 kDa band, consistent with previous reports of the βLRP-1 light subunit. Identification of both protein subunits in the
tissue samples supports functional LRP-1 expression in SCN. The relative intensity of the αLRP-1 515 kDa band was higher at ZT16 and ZT23 than at ZT6 (one-way ANOVA: n = 5-6, F(3,16) = 5.697, p = 0.0226), indicating a diurnal variation in LRP-1 expression (Figure 3.1a). Interestingly, the 85kDa βLRP-1 did not exhibit the same pattern: instead, its expression decreased in correlation with the time slices were maintained in vitro (one-way ANOVA: n = 5, F(3,16) = 0.9386, P = 0.0026) (Figure 3.1b). We also assessed LRP-1 phosphorylation on the β subunit using a phospho-specific LRP-1 antibody recognizing pY4507. In vitro LRP-1 phosphorylation at Y4507 did not exhibit a circadian rhythm, and also decreased across time in vitro, corresponding with the changes observed for the LRP-1 85kDa subunit (one-way ANOVA: n = 3, F(3,8) = 0.5285, P = 0.026) (Figure 3.1c). Collectively, these results suggest that LRP-1 expression exhibits both diurnal changes and time-in vitro dependent changes in vitro, and that the heavy subunit and light subunit have differing expression patterns.

3.4.2 Inhibiting LRP-1 prevents glutamate induced phase shifts in vitro

To investigate the role of LRP-1 in circadian clock phase shifting, we assessed the effect of inhibiting LRP-1 on glutamate-induced phase shifts of neuronal activity recorded in mouse SCN slices. First, we treated SCN slices with 1 mM glutamate +/- RAP, then conducted extracellular recordings of neuronal activity on the second day in vitro to determine the time of peak firing rate, which reflects the phase of the underlying circadian clock. RAP is a potent inhibitor of the LDL receptor family that is commonly used to assess LRP-1 function (Prasad et al 2015). Consistent with previous reports, 1 mM glutamate applied for 10 minutes at ZT16 induced a phase delay in SCN peak neuronal activity, with a mean phase shift of -3.08 +/- 0.51 h; n = 3 (Figure 3.2a). Applying 500 nM RAP concurrently with glutamate prevented these glutamate-induced phase delays, with a mean phase shift of -0.56 +/- 0.26 h, n = 4 (t-test glutamate vs glutamate + RAP: n = 3 to 4, t3 = 4.826, p = 0.0048) (Figure 3.2b). Additionally, RAP inhibition of glutamate-induced phase delays was dose dependent, and 100 nM was sufficient to prevent phase delays (one-way ANOVA: n = 3 to 4, F(3,9) = 0.1851, p = 0.0027) (Figure 3.2g). 500 nM RAP applied alone for 20 minutes at ZT16 had no effect on mean time of peak neuronal activity (one sample t-test of 500 nM RAP vs 0: n = 3, t(2) = 1.155, p = 0.3675).
Figure 3-1. LRP-1 expression and phosphorylation in SCN in vitro.

LRP-1 is expressed in mouse SCN in vitro, and the α and β subunits are expressed differentially. Proteins extracted from the SCN were subjected to western blotting analysis. (A) Representative images showing αLRP-1 band at ~515 kDa, and actin load control (~42 kDa), and corresponding histogram showing αLRP-1 relative to actin, normalized to ZT6 in WT SCN. One-way ANOVA p = 0.0485, n = 6. (B) Representative images and corresponding histogram showing βLRP-1 (~85 kDa) and actin. One-way ANOVA p = 0.0002, n = 5. (C) Representative images and corresponding histogram for pLRP-1 Y4507 (~85 kDa) and actin. One-way ANOVA p = 0.0026, n = 3-6.
Figure 3-2. Inhibiting LRP-1 prevents glutamate-induced phase shifts at ZT16 and ZT23.

Inhibiting LRP-1 prevents glutamate-induced phase shifts at ZT16 and ZT23. Shown here are the 2-hr means +/- SEM of the spontaneous neuronal activity recorded in single experiments, double bar denotes time of treatment, dashed bar indicates time of peak in neuronal activity rhythm in non-treated control. (A) Glutamate (1 mM) treatment at ZT16 induced a -3.08 +/- 0.51 h phase delay. (B) 100 nM RAP prevented glutamate-induced phase delays. (C) Shown are mean +/- SEM phase shift induced by treatments at ZT16. * p < 0.05 (D) Glutamate (1 mM) treatment at ZT23 induced a +2.66 +/- 0.21 h phase advance. (E) 500 nM RAP prevented glutamate-induced phase advances. (F) Shown are mean +/- SEM phase shift induced by treatments at ZT23. * p < 0.05 (G) ZT16 RAP dose response curve. (H) ZT23 RAP dose response curve.
Figure 3-2 continued
Because RAP can inhibit multiple members of the LDL receptor family, we addressed LRP-1 specifically by repeating the experiment using the anti-LRP-1 antibody R2629 antibody, which selectively inhibits LRP-1 (Mikhailenko et al., 2001). Concurrent application of 75 μg/mL R2629 with 1 mM glutamate resulted in a mean phase shift of 0.17 +/- 0.17 h, n = 3, indicating that selective inhibition of LRP-1 prevented glutamate-induced phase delays (t-test comparing glutamate vs glutamate + R2629: n = 3, t(6.091) = 6.091, p = 0.0037) (Figure 3.2c). Again, 75 μg/mL R2629 applied alone for 40 minutes at ZT16 had no effect on the mean time-of-peak neuronal activity (one sample t-test compared to 0: n = 3, t(0.4804) = 0.4804, p = 0.6784) (Figure 3.2c).

We repeated this series of treatments at ZT23 to address whether LRP-1 also influences phase advances. As previously reported, 1 mM glutamate treatment resulted in a phase advance of neuronal activity rhythms, with a mean shift of 2.66 +/- 0.21 h, n = 4 (Figure 3.2d). Concurrent application of 500 nM RAP with 1 mM glutamate blocked these phase advances, with a mean phase shift of 0.67 +/- 0.3 h, n = 3 (t-test comparing glutamate vs glutamate + RAP: n = 3, t(5.601) = 5.601, p = 0.0025) (Figure 3.2e). RAP inhibited glutamate-induced phase advances at ZT23 in a dose dependent manner, and 500 nM RAP was necessary to block phase advances (Figure 3.2g) (one-way ANOVA: n = 3 to 4, F(3,9) = 10.04, P = 0.0031). Selectively inhibiting LRP-1 with 75 μg/mL R2629 during 1 mM glutamate treatment also prevented phase advances, with a mean phase shift of 0.67 +/- 0.33 h, n = 3 (t-test comparing glutamate vs glutamate + R2629: n = 3 to 4, t(5.304) = 5.304, p = 0.0032) (Figure 3.2f). Neither RAP nor R2629 applied alone at ZT23 had any effect on mean time-of-peak (Figure 3.2f). Collectively, these results demonstrate that LRP-1 is required for glutamate-induced circadian clock phase shifting in mouse SCN in vitro.

3.4.3 RAP prevents glutamate-induced phase delays in tPA<sup>−/−</sup> mouse SCN tissue

Given LRP-1’s promiscuity, a variety of mechanisms could be responsible for its role in circadian clock phase shifting. LRP-1’s interactions with the plasminogen activating system were a likely candidate for LRP-1 involvement in the SCN. We assessed whether or not tPA is required for LRP-1’s role in phase shifting by repeating the RAP inhibition experiments in SCN slices from tPA<sup>−/−</sup> mice. As above, SCN containing brain slices were treated concurrently with 1 mM glutamate +/- 500 nM RAP and neuronal activity was recorded the following day. As previously reported (Cooper et al., 2017), 1 mM glutamate
applied at ZT16 induced a -3.13 +/- 0.65 h (n=4) phase shift in tPA+ SCN brain slices (Figure 3.3). Concurrent application of 500 nM RAP with 1 mM glutamate blocked these phase delays, with a mean phase shift of a -0.58 +/- 0.35 h (n=6) (t-test comparing glutamate vs glutamate + RAP: t(8) = 3.761, p = 0.0055), while 500 nM RAP applied alone had no effect (mean phase shift = -0.167 +/- 0.167, n = 3). These results suggest that the inhibition of glutamate-induced phase shifts by RAP does not involve preventing a tPA-LRP-1 interaction.

3.4.4 RAP treatment does not influence maturation of BDNF

To address the involvement of the tPA-BDNF-TrkB signaling cascade in another way, we used western blotting to assess the effects of glutamate and RAP on BDNF maturation in SCN tissue. Brain slices containing the SCN were treated with 1 mM glutamate, 1 mM glutamate + 100 nM RAP, or 100 nM RAP. They were collected after a 5 min recovery period in normal EBSS and compared to no treatment controls collected at the same time. There were no significant differences in total proBDNF (one-way ANOVA: n = 6 to 10, F(3,27) = 1.524, p = 0.6566), total mBDNF (one-way ANOVA: n = 6 to 10, F(3,27) = 0.1511, p = 0.9132), or the mBDNF/proBDNF ratio (one-way ANOVA: n = 6 to 10, F(3,27) = 0.2497, p = 0.9838) across any of the treatments (Figure 3.4). Collectively, these results suggest that not only does RAP not acutely change BDNF maturation independently, but also that there are no acute changes in BDNF maturation following glutamate treatment that require LRP-1.

3.4.5 RAP treatment does not change TrkB receptor phosphorylation on Y680

As a final way to assess the tPA-BDNF-TrkB signaling cascade, we used western blotting to assess whether RAP treatment influences Trk receptor phosphorylation on Y680/681, which also recognized the homologous residues Y706/707 on TrkB, as an indicator of Trk receptor activation (Huang & Reichardt, 2003; Reichardt, 2006)(Wong et al 2008. As before, SCN slices treated with 1 mM glutamate, 1 mM glutamate + 100 nM RAP, or 100 nM RAP were compared to untreated control slices at ZT16. Once again, we found no significant differences across any treatments (One-way ANOVA: n = 3, F(3,8) = 0.6715, p = 0.864) (Figure 3.5). These results suggest that inhibiting LRP-1 does not influence TrkB receptor activation, and thus they reinforce the conclusion that the tPA-
Figure 3-3. RAP blocks phase shift in tPA<sup>−/−</sup> SCN brain slices.

100 nM RAP prevents glutamate-induced phase delays in brain slices prepared from tPA<sup>−/−</sup> mice. Shown are mean +/- SEM phase shifts induced by treatments indicated applied to SCN slices prepared from tPA<sup>−/−</sup> mice. Phase delays plotted as negative values. * p = 0.0055, n = 3 to 6.
Figure 3-4. RAP treatment does not influence TrkB receptor phosphorylation.

TrkB receptor phosphorylation on Y680/681 does not change with 1 mM glutamate, 1 mM glutamate + 100 nM RAP, or 100 nM RAP treatment at ZT16. (A). Shown are representative blots of pTrkB (~140 kDa) and actin load control (~42 kDa). (B). Mean +/- SEM pTrk(680/681) relative to actin, normalized to control. No significant differences in one-way ANOVA, n = 3.
Figure 3-5. RAP treatment does not influence BDNF maturation in SCN.

BDNF maturation does not change with 1 mM glutamate, 1 mM glutamate + 100 nM RAP, or 100 nM RAP treatment at ZT16. (A) Shown are representative blots of proBDNF (~32 kDa), mBDNF (~14 kDa), and actin load control (~42 kDa) from WT SCN which were treated as indicated. (B) Histogram showing proBDNF as a ratio to actin, normalized to control. (C) Histogram showing mBDNF as a ratio to proBDNF, normalized to control. (D) Histogram showing mBDNF as a ratio to actin, normalized to control. No significant differences in one-way ANOVA: n = 6 to 9.
BDNF-TrkB cascade is not the primary mechanism through which LRP-1 influences phase regulation of the SCN circadian clock.

3.4.6 RAP treatment influences NR2B phosphorylation in SCN

Having eliminated the tPA-BDNF-TrkB cascade as the primary mechanism of LRP-1 action in SCN clock phase shifting, we turned our focus to more direct interactions with NMDAR. LRP-1 influences glutamate signaling partially by controlling NMDAR surface localization (Maier et al., 2013), and therefore this could underlie LRP-1 functions in the SCN. We investigated whether RAP influences NR2B phosphorylation at Y1472 and S1480, which are residues that influence NMDAR surface localization (Lim et al., 2002; Prybylowski et al., 2005; Sanz-Clemente et al., 2013). Phosphorylation on Y1472 is associated with increased localization of NMDARs to the cell membrane, and phosphorylation on NMDAR S1480 is associated with increased internalization of NMDARs. SCN slices treated with 100 nM RAP for 20 minutes at ZT16 were compared to no-treatment controls via western blotting using phospho-specific antibodies for NR2B Y1472 and S1480. 100 nM RAP treatment resulted in a decrease in NR2B S1480 phosphorylation (t-test control vs 100 nM RAP: n = 4, t(6) = 8.15, p = 0.002), and no significant change in NR2B Y1472 phosphorylation (t-test control vs 100 nM RAP: n = 4, t(5) = 0.3814, p = 0.7186) (Figure 3.6). Thus, these data suggest that inhibiting LRP-1 decreases NR2B phosphorylation on S1480.

3.4.7 Assessing effects of inhibiting LRP-1 on pCaMKII

To further explore whether LRP-1 modulates glutamate-induced phase shifts by influencing NMDAR signaling, we investigated activation of CaMKII, an important early step in the glutamate phase shifting cascade, by assessing glutamate-induced phosphorylation of CaMKII T286 (Giese et al., 1998; Fukunaga et al., 2002). Initially we compared untreated SCN brain slices with brain slices treated with 1 mM glutamate at ZT16. Subsequently, we ran a more comprehensive set of experiments that included treating SCN-containing brain slices at ZT 16 with 1 mM glutamate, 1 mM glutamate + 100 nM RAP, 100 nM RAP, or no treatment. In both cases we collected the tissue 5 minutes after treatment, and conducted western blotting assays. Consistent with previous reports (Yokota et al., 2001), our initial experiments confirmed that glutamate (1 mM) increases
Figure 3-6. RAP treatment changes NMDAR phosphorylation patterns in mouse SCN in vitro.

RAP treatment reduces phosphorylation of NR2B subunits at S1480, and does not change phosphorylation on NR2B Y1472. (A) Shown are representative blots of pY1472 (~180 kDa) and load control actin (~42 kDa). (B) Histogram showing mean +/- SEM pY1472/actin, normalized to first control on each blot. No significant differences in t-test, n = 3 to 4. (C) Representative blots of pS1480 (~180 kDa) and load control actin. (D) Mean +/- pS1480/actin, normalized to control on each blot. * p = 0.002, n = 4.
pCaMKII T286 in the SCN (t-test: n = 3, t(4) = 4.716, p = 0.0092,) (Figure 3.7a-b). However, in subsequent experiments comparing all 4 experimental conditions the relative expression of pCaMKII was highly variable, so we found no significant differences across treatments (One-way ANOVA: n = 5 to 6, F(3,19) = 0.3477, p = 0.8412, F_3 = 0.2771) (Figure 3.7c-d).

3.5 Conclusion

Here, we present evidence that LRP-1 is expressed in the SCN, and that it is necessary for glutamate-induced phase resetting of the circadian clock in the mouse SCN in vitro. Additionally, we investigated several prominent LRP-1 interacting partners as potential mediators of LRP-1 effects, with a specific focus on the plasminogen activating cascade and intracellular signaling molecules downstream of NMDAR activation. We find that LRP-1’s role in the SCN is independent of tPA, acute changes in BDNF maturation, and TrkB receptor phosphorylation, effectively eliminating tPA proteolytic activity as a central mediator of LRP-1 effects on phase shifting the circadian clock. We find that inhibiting LRP-1 influences NMDAR phosphorylation patterns by reducing phosphorylation on NR2B S1480, but we were unable to determine whether inhibiting LRP-1 influences pCaMKII phosphorylation patterns.

LRP-1 is widely expressed throughout the central nervous system, but a direct assessment of its expression patterns in the SCN had not previously been conducted (Lillis et al., 2005). Western blotting shows LRP-1 expression in the SCN, as evidenced by the presence of both the 515 kDa αLRP-1 and 85 kDa βLRP-1 subunits. Surprisingly, we find differential expression patterns for the two subunits of LRP-1. Expression of the extracellular αLRP-1 is higher at night than during the day, a pattern that persists to the second day in vitro. Meanwhile, expression of the membrane-spanning βLRP-1 subunit decreases in correlation with the time SCN brain slices are maintained in vitro. It is unclear at this point how this is achieved and what physiological relevance it would have in vivo. LRP-1 is transcribed as a full-length 600 kDa protein and then cleaved in the endoplasmic reticulum into a 515 kDa αLRP-1 and 85 kDa βLRP-1, which remain non-covalently associated on the cell surface (Lillis et al., 2005). The decrease in βLRP-1 over time in vitro could be a response to tissue slicing injury. There is evidence that LRP-1 responds to crush-injured rat sciatic nerves in the peripheral nervous system (Flutsch et al., 2016).
Figure 3-7. Glutamate increases CaMKII phosphorylation at T286 in SCN.

(A). Representative images of pCaMKII and actin bands from control and glutamate treated SCN slices, demonstrating glutamate-induced increase in CaMKII phosphorylation. (B) Mean +/- SEM pCaMKII/actin normalized to control. *p = 0.0092, n = 3. (C) & (D): Representative images of pCaMKII, total CaMKII, and actin bands from SCN slices left untreated (C), or treated with 1 mM glutamate (G), 1 mM glutamate + 100 nM RAP (GR), or 100 nM RAP demonstrating variability in pCaMKII levels. (E). Mean +/- SEM pCaMKII/actin normalized to no-treated control. No significant differences in One-way ANOVA.
Additionally, this response to injury could lead to increased internalization of the β subunit, as LRP-1 could be serving a scavenging role in response to released proteins in the extracellular space. This is reminiscent of the time-in vitro dependent increases in uPA enzymatic activity observed previously in our lab (Cooper et al., 2017). These results underscore the importance of controlling for multiple timing variables in circadian studies, while further implicating the plasminogen activating cascade in a group of regulated neuronal responses to injury (Mori et al., 2001; Flutsch et al., 2016; Diaz et al., 2017).

In contrast, the rhythm in αLRP-1 expression suggests there may be a circadian influence on LRP-1 in the SCN. This is a first report of diurnal variations in LRP-1 expression, but interestingly Lrp-1 was identified as a circadian oscillating gene in liver (Yan et al., 2008). αLRP-1 can exist in the extracellular space as “shed” LRP-1, which is generated by proteolytic cleavage from the membrane-bound β subunit (Quinn et al., 1997; Etique et al., 2013). The precise function of shed LRP-1 remains elusive, but it may serve to sequester LRP-1 ligands in the extracellular space, thus controlling their activity (Etique et al., 2013). It is somewhat perplexing that αLRP-1 expression increases during the subjective night while βLRP-1 expression decreases. One possible explanation for these results is that there are circadian changes in proteolytic cleavage of the extracellular domain, allowing αLRP-1 to accumulate extracellularly while βLRP-1 is internalized/degraded (Figure 3.8). LRP-1 shedding can be mediated directly or indirectly by a variety of extracellular proteases, and at least on protease associated with increased LRP-1 shedding, tPA, is known to have higher activity in the SCN at night than during the day (Mou et al., 2009b; Etique et al., 2013; Cooper et al., 2017). Although LRP-1 has been shown to recycle with near 100% efficiency (Van Leuven et al., 1980; Willingham et al., 1980; Dickson et al., 1981), it is possible that following injury a different scenario exists. A second possibility is that our data reflect changes in LRP-1 localization to lipid rafts (Figure 3.8). LRP-1 localization on the cell surface is dynamic, and it can localize differentially to lipid rafts or clathrin coated pits depending on ligand or receptor interactions (Wu & Gonias, 2005). Association in lipid rafts could influence LRP-1 solubilization during protein extraction, which would mean our blots would reflect different amounts of LRP-1 solubilized rather than a difference in expression. Although the source of the differences in patterns of expression in the αLRP-1 vs βLRP1 are unclear, these results suggest both diurnal regulation and injury regulation of LRP-1 expression in the
Figure 3-8. Potential models underlying differential α and β LRP-1 expression patterns.

Option 1:  1) SCN slices are made in the morning, interrupting normal expression patterns in the SCN with a slicing injury. 2) An increase in LRP-1 expression combined with an increase in proteolytic activity as the SCN transitions into subjective night results in an increase of shed αLRP-1 in the extracellular space. 3) A concurrent (or slightly delayed) increase in LRP-1 internalization results in increased degradation of LRP-1, effectively reducing the amount of βLRP-1 detected. This decrease persists into the second day in vitro. Option 2: 1) In the subjective day, LRP-1 could localize outside of lipid rafts. 2) As the SCN transitions to subjective night, and perhaps in response to slicing injury, there is an increase in extracellular domain shedding combined with (3) a localization of LRP-1 to lipid rafts. The result of this is more αLRP-1 detected on western blots, and a reduction in the ability to solubilize βLRP-1 from the lipid rafts, resulting in reduced βLRP-1 in protein extracts. The purpose of these two models is not to provide definitive answers for what is happening in the SCN, but rather to provide possible explanations for the seemingly impossible finding of differential patterns of immunolabeling in the LRP-1 subunits. To fully understand LRP-1 expression in the SCN, it will be important to complete experiments without the complication of slicing injury.
SCN, and thus further investigation into LRP-1 expression patterns is warranted. Importantly, there is a large amount of time between ZT6 (4 h in vitro) and ZT16 (6 h in vitro) that is unaccounted for, and in which there could be changes that set the stage for the differential expression patterns we observed here.

Along with assessing total LRP-1 expression, we also assessed LRP-1 phosphorylation on Y4507 using a phospho-specific antibody. Phosphorylation on this residue is correlated with changes in LRP-1 endocytosis and signaling events, and thus could be indicative of changes in LRP-1 activity rather than expression (Betts et al., 2008; Guttman et al., 2009). We observe no circadian rhythm in LRP-1 Y4507 phosphorylation and decreases that correlate with time in vivo, which is consistent with our observations for βLRP-1, suggesting no differential phosphorylation in our system.

In an assessment of LRP-1 function in the SCN, we find that LRP-1 is required for glutamate-induced phase shifting. We used two methods of inhibition to assess LRP-1: non-selective inhibition with RAP and selective inhibition with antiLRP-1 antibody (R2629), and both completely abrogate both early night glutamate-induced phase delays and late night phase advances. While our data support LRP-1 involvement, RAP inhibits multiple members of the LDLR family, and therefore it would be interesting to see if additional LDLR family receptors are also involved in phase shifting. At both ZT16 and ZT23, the inhibition by RAP is dose-dependent, although ~ 100nM RAP fully inhibits the phase delays while >300nM RAP is needed to inhibit the phase advances. Phase delays and phase advance involve different signaling mechanisms, and differential expression of LRP-1 interacting proteins between early and late night could contribute to the differences in dose responsiveness.

A plethora of evidence linking LRP-1 to the plasminogen activating system and Trk receptors, and evidence supporting a critical role for the plasminogen activators in SCN circadian clock phase shifting led us to hypothesize that LRP-1 modulation of circadian clock phase shifting involves interactions with this pathway. We addressed this possibility in three ways. First, we used tPA+ mice to investigate whether tPA is necessary for RAP to inhibit glutamate-induced phase shifting, and found that in tPA+ mouse SCN brain slices RAP is still able to block these phase shifts. This indicates that LRP-1 modulation of the SCN circadian clock does not depend on interactions with tPA, although it doesn’t exclude the possibility that tPA and LRP-1 interact in the SCN and have other effects relevant to
the circadian clock. It appears that compensatory changes occur in tPA<sup>−/−</sup> mice in response to the absence of tPA: despite tPA’s critical role in blood clotting, tPA<sup>−/−</sup> mice exhibit no severe clotting phenotype (Carmeliet et al., 1994). In regards to the SCN circadian clock, tPA<sup>−/−</sup> mice have minimal circadian phenotypic deficits, and uPA appears to serve a compensatory role enabling glutamate-induced phase shifts (Cooper et al., 2017; Krizo et al., 2018). It is possible that similar compensation masks our ability to detect a role for the tPA/LRP-1 interaction in these animals. And finally, the tPA<sup>−/−</sup> mice used in this study still express a portion of the tPA gene, thus non-proteolytic tPA effects cannot be excluded (Carmeliet et al., 1994).

Second, to more directly assess the influence of LRP-1 on the tPA-dependent proteolytic cascade, we assessed the effects of inhibiting LRP-1 on BDNF maturation. In the SCN, BDNF acts to gate glutamate induced phase shifts downstream of tPA proteolytic activity (Liang et al., 1998; Liang et al., 2000; Mou et al., 2009b). Thus, if LRP-1 acts through modulating tPA proteolytic activity in the SCN, we would expect to see acute changes in mBDNF generation following LRP-1 inhibition. Instead, we find no changes in the relative amount of mBDNF, proBDNF, or the mBDNF/proBDNF ratio, indicating that inhibiting LRP-1 does not influence BDNF maturation in the SCN <i>in vitro</i>.

Lastly, we investigated the effects of inhibiting LRP-1 on Trk receptor phosphorylation as a marker of TrkB receptor activation. BDNF binding to TrkB receptor causes it to dimerize and autophosphorylate several sites, including Y705Y706 (which is homologous to Y680/680 on TrkA receptors), Y515, and Y816 (Poo, 2001; Huang & Reichardt, 2003; Reichardt, 2006). This leads to signal transduction and activation of two signaling cascades (MAPK/pCREB, and phosphatidylinositol 3-kinases (PI<sub>3</sub>K)/protein kinase B (AKT)) (Cardenas-Aguayo Mdel et al., 2013). In the SCN, TrkB acts to gate glutamate induced phase shifting following mBDNF binding (Allen et al., 2005). We find that inhibiting LRP-1 using RAP does not change Trk receptor phosphorylation on Y680/681. Thus, by assessing phase shifting in tPA<sup>−/−</sup> mice, BDNF maturation, and TrkB receptor phosphorylation following RAP treatment we have convincingly demonstrated that the tPA-BDNF-TrkB receptor cascade is not primarily responsible for RAP’s ability to inhibit glutamate-induced phase shifting. That said, these data do not rule out the possibility that interactions between these proteins in the SCN mediate other clock functions.
A second prominent mechanism through which LRP-1 modulates glutamate responses in other systems, particularly Schwann cells, is by controlling NMDAR surface localization, as evidenced by aberrant NMDAR localization patterns in LRP-1 NPxY mutant cell lines (Maier et al., 2013). Regulation of NMDAR localization is complex and mediated by a variety of events, but a major contributor is phosphorylation on two NR2B residues, Y1472 and S1480. Phosphorylation on NR2B Y1472 disrupts interactions with clathrin coated pits (Prybylowski et al., 2005; Chen & Roche, 2007) and is associated with increased membrane localization of NR2B containing NMDARs, while phosphorylation on S1480 disrupts interactions with PSD95, thus increasing NMDAR internalization (Chung et al., 2004). We assessed the influence of inhibiting LRP-1 on phosphorylation of both residues in SCN brain slices. We find that RAP treatment does not change phosphorylation on Y1472, but decreases phosphorylation on S1480. Given the model above, these changes could suggest an increase of NMDAR on the cell surface and thus enhanced rather than decreased NMDAR signaling. However, it is important to note that NMDAR localization can be controlled independently of these phosphorylation patterns. For example, NR2B phosphorylation patterns in Schwann cells are inconsistent with observed changes in NMDAR localization, and suggested that the increases in NR2B at the cell surface could be a direct effect of reduced LRP-1 internalization rate (Maier et al., 2013). Regardless, these results suggest LRP-1 influences NMDAR localization and/or other signaling properties in the SCN. It will be important to assess changes in NMDAR and LRP-1 surface localization in the future.

Finally, we assessed the influence of LRP-1 on CaMKII phosphorylation, which is a key mediator of glutamate signaling downstream of NMDAR activation. Glutamate induces CaMKII phosphorylation acutely in the SCN, and inhibiting CaMKII activation prevents circadian clock phase shifts (Fukushima et al., 1997; Yokota et al., 2001; Agostino et al., 2004). CaMKII interacts with the intracellular domain of LRP-1, and is a key mediator of LRP-1 effects on axon guidance in the peripheral nerve growth cone and thus it could be a key mediator of LRP-1 function in the SCN (Guttman et al., 2009; Landowski et al., 2016). We found that treating SCN slices with 1 mM glutamate increased CaMKII phosphorylation on T286, but in subsequent experiments to assess changes following RAP inhibition we were unable to detect any significant changes, which may be due to high variability in pCaMKII across samples. Across independent experiments, the
patterns of phosphorylation were completely opposite of one another. The SCN is a heterogeneous structure, and thus regional differences may be a source of this variability. Assessing CaMKII activation using immunohistochemistry could provide more conclusive answers.

The involvement of LRP-1 and potential rhythms in expression may provide some clarity to some of our previous data. In investigating tPA and uPA expression and activity in the SCN, we found different patterns for both proteases (Cooper et al., 2017). tPA protein expression was rhythmic in the SCN, but we found no changes in tPA total proteolytic activity. A rhythm in αLRP-1 could provide a mechanistic explanation for this. It is possible that increased night-time αLRP-1 binds to tPA, which could serve either to sequester tPA reducing its activity, or to act as a co-receptor increasing its activity. Regardless of the function outcome, these findings were generated from the non-reducing/non-denaturing conditions of gel zymography, meaning that tPA complexed to αLRP-1 may not dissociate on the gels. For uPA, we found its protein expression to be constant, but its proteolytic activity increased during the time the tissue was maintained in vitro. This correlates with the decrease we see in LRP-1 β subunit, suggesting that both changes are a response to tissue injury, with related or independent of each other. Teasing out these injury response mechanisms would be an interesting question, but is beyond the scope of this research. Additionally, LRP-1 expression in the SCN may be better assessed in a system without the consequence of injury.

Collectively, the data presented here demonstrate that LRP-1 is necessary for glutamate-induced phase-shifting of the SCN circadian clock, and suggest that it may be a mediator of daily iterative metaplasticity in the SCN. This study adds to accumulating evidence that changes in the extracellular space are important for circadian clock regulation. Through its endocytic and signaling activities, LRP-1 acts as a sensor and regulator of the extracellular space. It communicates changes in the extracellular environment via signal transduction, and can regulate the concentrations of its ligands in the extracellular space through endocytic functions. More research is necessary to determine the mechanisms underlying LRP-1 function in the SCN, but given its large and diverse group of ligands and complex endocytic and intracellular responses, it is likely that multiple interacting partners will be involved. Underscoring LRP-1’s complexity, we found evidence excluding many of the canonical pathways central to both SCN function and
LRP-1 mechanisms. The primary connections we found are, first, that RAP prevents a glutamate response in the SCN that relies largely on NMDAR activity, and second RAP induces a decrease in NR2B S1480 phosphorylation. It seems most likely that LRP-1 may be attenuating the NMDAR signals, and it has been found that LRP-1 influences other calcium channels, such as AMPARs and N-type calcium channels as well (Kadurin 2017, Gan 2014). Addressing whether this is happening in the SCN will be an important next step, and addressing the mechanisms through which LRP-1 can control calcium channels is another salient question. An important next step will be to assess what ligands are important for LRP-1 function in the SCN. Additionally, it will also be important to tease apart the contributions of LRP-1 endocytosis vs signaling events in the mediation of glutamate responses in the SCN. Use of neuronal specific LRP-1 knockout and LRP-1 NPxY mutant mice may assist in answering some of these questions.
CONCLUSION: AT THE INTERSECTION OF LRP-1 AND PLASMINOGEN ACTIVATORS - POTENTIAL MECHANISMS
4.1 uPA and LRP-1: Contributions to SCN circadian clock phase regulation

Herein, we have expanded on the knowledge of proteins related to the plasminogen activating cascade and how they participate in the process of phase shifting the mammalian circadian clock in the SCN. Although plasminogen activation was originally studied for its role in the vascular system, many studies have identified its members, including tPA, uPA, and LRP-1, as neuromodulators within the brain (Fernandez-Monreal et al., 2004; Jeanneret & Yepes, 2017). Evidence demonstrating the involvement of tPA in SCN circadian clock phase shifting has defined a role for this pathway in modulating neuronal plasticity in the SCN and suggested that tPA-interacting partners could also influence SCN neuronal activity (Mou et al., 2009a). The results presented here are the first linking both uPA and LRP-1 to the SCN circadian clock.

Initially, we found that although inhibiting tPA with PAI-1 prevents glutamate-induced phase shifts in vitro, tPA−/− mice exhibit no severe circadian deficits. They exhibit entrained behavioral activity rhythms in vivo and neuronal activity rhythms in vitro that phase shift in response to light and glutamate pulses, respectively. The only phase shifting deficit we observed is an increase in the time needed to entrain to a reversed light dark cycle in the tPA−/− mice. The tPA−/− mice also exhibit a decrease in nocturnal wheel-running activity, changes in activity patterns with food entrainment, and an increase it time needed to entrain to a 6 hr advancement of LD cycle in vivo (Krizo et al., 2018). We present evidence that uPA compensates for the loss of tPA in tPA−/− mice, enabling glutamate-induced phase shifts in neuronal activity rhythms in vitro. Interestingly, this functional compensation involves distinct cellular signaling mechanisms, as evidence supports tPA but not uPA acting through a plasmin-BDNF dependent mechanism. Thus, the processes underlying uPA’s compensatory processes remain unclear.

Second, we found that LRP-1 is necessary for glutamate-induced phase shifts of the mouse SCN circadian clock in vitro. Because tPA and LRP-1 act in concert to influence neuronal plasticity in other regions, we hypothesized that these interactions would be important for LRP-1’s role in phase shifting (Martin et al., 2008). However, we find that tPA is not necessary for LRP-1’s permissive actions in glutamate-induced phase shifting. Since NMDARs are a central mediator of glutamate-induced phase shifting in the
SCN, and LRP-1 can modulate NMDAR activity, we also focused our attention on the intersection between LRP-1 and NMDAR (Ebling, 1996). We find that inhibiting LRP-1 influences NMDAR phosphorylation patterns, which implicates changes in NMDAR cell-surface localization. Finally, we assessed CaMKII phosphorylation as a marker of signaling events downstream of NMDAR activity in the SCN, but we were unable to determine if inhibiting LRP-1 influences CaMKII activation. Collectively, these data implicate LRP-1 as an important regulator of clock phase shifting, but as with uPA, the mechanism(s) remain elusive.

In this research we also evaluated uPA and tPA expression and proteolytic activity in the SCN across the circadian day, and LRP-1 expression and phosphorylation patterns. We find evidence of circadian rhythms in tPA expression but not proteolytic activity, no rhythms in uPA expression or proteolytic activity, and potential diurnal variations in αLRP-1 but not βLRP-1 subunits. Additionally, uPA activity and βLRP-1 expression exhibit changes that correlate with the time slices are maintained in vitro, suggesting that a response to tissue injury may occlude an accurate view of in vivo expression patterns in the SCN. Importantly, these results demonstrate roles for both uPA and LRP-1 in the SCN phase regulation, but both halves of the story leave open major questions regarding the underlying mechanisms. In this chapter, I discuss potential overlap between these two seemingly independent studies, and incorporate them into a model linking extracellular events to the more extensively studied intracellular circadian clock mechanisms. And finally, I highlight ways the plasminogen activators and LRP-1 may act together to influence neuroplasticity in the SCN.

4.2 Bridging the gap – common signaling mechanisms of tPA, uPA, and LRP-1

tPA, uPA, and LRP-1 all modulate neuronal activity, and can do so both independently and through intersecting processes that aren’t fully understood and that are likely both cell type and brain region specific. Points of overlap include regulation of the extracellular matrix (ECM), influence on other extracellular proteases such as MMPs, interactions with NMDARs, interactions with integrins, associations with uPAR, and activation intracellular signaling pathways. Of particular importance to these studies are
interactions between LRP-1 and uPA: uPA can bind to LRP-1 both alone and when bound by PAI-1, and one intersection between LRP-1 and uPA are their interactions with uPAR.

4.2.1 Extracellular matrix plasticity in the brain

The ECM is an important modulator of neuronal activity and a source of overlap for many tPA, uPA, and LRP-1 dependent functions. The ECM is a network of secreted molecules interacting through protein-protein and protein-carbohydrate binding in the extracellular space (Senkov et al., 2014; Jayakumar et al., 2017). ECM components include chondroitin sulfate proteoglycans, heparin sulfate proteoglycans, collagen, elastin, laminin, fibronectin, and hyaluronic acid (Senkov et al., 2014; Jayakumar et al., 2017). Additional secreted proteins, including growth factors, proteases, thrombospondins, tenascin C and R, reelin, vitronectin, PAI-1, and chemokines can bind and modify the ECM (Senkov et al., 2014; Jayakumar et al., 2017). Cell surface proteins and receptors, including integrins, syndecans, agrin, lipoprotein receptors, and tetraspanins, also interact with the ECM (Kerrisk et al., 2014). The ECM is considered a 4th component of a tetrapartate synapse model, which consists of pre- and post-synaptic neuronal terminals surrounded by a network of astrocytes and ECM molecules (Dityatev & Rusakov, 2011; Smith et al., 2015). During development, the ECM acts to guide neuronal migration and synapse formation, while in adulthood it is thought to stabilize and strengthen connections (Pavlov et al., 2004). Moreover, remodeling of the ECM in adulthood can contribute to the structural rearrangements necessary to change synaptic strength associated with long term potentiation and long term depression (Senkov et al., 2014; Cooper, Submitted). ECM remodeling is largely mediated by proteolytic cleavage, and can occur in both physiological and pathological contexts (Lu et al., 2012).

Several lines of evidence support the concept that the ECM is an important part of SCN plasticity. One intriguing finding is that there are daily rhythms in the ultrastructure of the SCN; in particular astrocytic processes invade and retract from synapses on a 24 hr cycle (Becquet et al., 2008). This suggests that structural changes, which depend heavily on ECM remodeling in other brain regions, are an important component of SCN daily plasticity. Additional support for ECM involvement comes from the identification of several ECM-interacting proteins in clock function. One class of ECM-interacting proteins that has been investigated in the SCN are cell adhesion molecules (CAMs), which are
membrane associated proteins that form adhesions with binding partners on adjacent cells, or with the ECM itself (Thalhammer & Cingolani, 2014). Cell adhesion molecules that have been implicated in SCN clock function include neural cell adhesion molecules (NCAMs), neurexins, neuroligins, ephrins, Eph receptors, and cadherins (Cooper, Submitted). As we will discuss below, the investigation of extracellular proteases including tPA, uPA, and MMPs, as well as our data demonstrating a role for LRP-1, adds to the evidence supporting a role for the ECM in the SCN.

4.2.2 Extracellular protease regulation of ECM: tPA, uPA, and MMPs

Many proteolytic enzymes cleave ECM macromolecules, including tPA, uPA, (Andreasen et al., 2000) and MMPs (Murphy & Nagase, 2008). Through their proteolytic activity, extracellular proteases influence the structure of the ECM, which in turn can influence the strength of synaptic connections and modify neuronal responses. One way tPA and uPA can influence ECM molecules is through plasmin-dependent functions (Ho-Tin-Noe et al., 2009). Plasmin degrades many ECM macromolecules, including laminin, fibronectin, and proteoglycans. Plasmin can also activate MMP’s, and indirectly affect additional ECM molecules in this way (Mignatti & Rifkin, 1996; Legrand et al., 2001). MMPs are a large family of zinc-dependent endopeptidases that can be secreted or membrane-bound, and can degrade a variety of ECM molecules, including collagen, gelatin, laminin, and fibronectin. tPA and uPA also influence the ECM by activating or releasing growth factors such as latent-transforming growth factor β and vascular endothelial growth factor from the ECM.

Our evidence demonstrating that plasminogen activation is important for SCN clock phase regulation also implicates ECM remodeling as a component of circadian timekeeping mechanisms. While the initial data supports a model whereby tPA acts via BDNF signaling to enable phase shifts, uPA appears to be acting through BDNF-independent mechanisms (Mou et al., 2009a; Cooper et al., 2017). Additionally, uPA compensation is not complete, as there are deficits in the ability of tPA−/− mice to re-entrain to an inverted light cycle, and a reduction in ability to phase advance in vivo (Krizo et al., 2018). Both of these findings raise the possibility that plasminogen activators act through multiple pathways to influence the SCN circadian clock. This type of redundancy in SCN in timekeeping mechanisms may have been evolutionarily selected for because problems
with circadian rhythms are maladaptive. Additional evidence supporting ECM involvement in the clock comes from research on MMPs in the SCN, which has found that inhibiting MMP2/9 induces night-time phase shifts. The mechanisms through which the MMPs modulate the SCN circadian clock appear to be complex, in that some of the inhibitor-induced phase shifts are independent of the plasminogen activating cascade, while others require plasmin. Additionally, there may be diurnal variations in MMP9 proteolytic activity in mouse and hamster SCN (Agostino et al., 2002; Abrahamsson, Submitted). Collectively, tPA, uPA, and MMP2/9 involvement in circadian clock phase shifts support the idea that extracellular proteases have diverse functions in the SCN, and ECM modification may be a source of overlap. Rhythms in protease expression or activity could correlate with rhythmic structural remodeling of ECM components, enabling the extension and retraction of astrocytic processes, thus adjusting synaptic connections on a 24 hr cycle.

4.2.3 Protease Inhibitors – unexplored partners

The activity of extracellular proteases can be regulated in two ways: by endogenous inhibitors and by receptor-mediated endocytosis to clear them from the extracellular space (discussed below). The plasminogen activators are inhibited by serpins (serine proteinase inhibitors), including plasminogen activator inhibitors (PAI)-1 and -2, neuroserpin, and protease nexin-1 (PN-1) (Huntington, 2011). PAI-1 is the main inhibitor of plasminogen activators in the vascular space, but is weakly expressed in the brain (Sawdey & Loskutoff, 1991; Masos & Miskin, 1997). PN-1 and neuroserpin are expressed throughout the brain, and neuroserpin is considered the predominant PA inhibitor in the nervous system (Osterwalder et al., 1996; Hastings et al., 1997; Krueger et al., 1997; Kvajo et al., 2004; Samson et al., 2008). MMPs are inhibited by tissue inhibitors of metalloproteinases (TIMPs) and adamalysins (a disintegrin and metalloproteinases, ADAMs) (Wojtowicz-Praga et al., 1997). α2-macroglobulin is another protease inhibitor that can act on many proteolytic enzymes, including tPA, uPA, plasmin, and MMPs (Rehman et al., 2013). Many of these protease inhibitors have been found to influence neuronal activity, which could involve protease inhibition or protease independent interactions with receptors (Lee et al., 2008). Additionally, some of these (including PAI1/2, TIMPs, α2-macroglobulin, and neuroserpin) bind to LRP-1, either independently
or in complex with their target, and can be endocytosed or stimulate signaling pathways (Lillis et al., 2008).

Although we have used these inhibitors to investigate tPA and uPA function in the SCN, we have not yet investigated the endogenous roles these inhibitors may be playing. Interestingly, PAI-1 mRNA and protein expression is rhythmic in the SCN in antiphase with tPA rhythms, with high expression in the day and low expression at night (Menger et al., 2005; Mou et al., 2009a). This suggests that PAI-1 may inhibit tPA activity during the day. Additionally, vitronectin, which stabilizes PAI-1 in its active conformation, is necessary for PAI-1’s inhibitory action on phase shifting, as PAI-1 doesn’t inhibit phase shifts in vitronectin knockout mice (VN−/−) (Mou et al., 2009a). Interestingly, expression of PAI-1 also exhibits circadian rhythms in the periphery, and is thought to be regulated by the TTFL, as CLOCK:BMAL heterodimers upregulate PAI-1 gene expression (Oishi et al., 2007). These rhythms in PAI-1 contribute to hypo-fibrinolysis during the early morning (Oishi et al., 2007). There is also preliminary data supporting neuroserpin expression and function in the SCN (Conner and Prosser, unpublished). Much more work is needed to elucidate the roles these protease inhibitors play in the SCN, and to what extent they contribute to circadian clock phase regulation.

4.2.4 LRP-1 regulation of extracellular proteases

LRP-1 regulates extracellular signaling through several mechanisms, including endocytic regulation of protease activity and interactions with ECM-associated proteins (Etique et al., 2013). First, through its endocytic activity it internalizes proteases, including uPA, tPA, MMP9 (Bu et al., 1992; Kounnas et al., 1993; Hahn-Dantona et al., 2001), MMP2, and MMP13 (Barmina et al., 1999; Yang et al., 2001). LRP-1 can internalize these proteins either before or after their inhibition by serpins or α2-macroglobulin (Strickland et al., 2002). This internalization can serve to regulate their proteolytic activity in the extracellular space. For example, astrocytes can internalize tPA in an LRP-1 dependent manner, and then recycle tPA back to the extracellular space through subsequent release (Casse et al., 2012b). The internalization of tPA is inhibited by glutamate (Casse et al., 2012b). Collectively, the cycle suggests that astrocytes control the amount of tPA in the extracellular space through an LRP-1 dependent mechanism (Casse et al., 2012b).
Although our data suggest that tPA is not required for LRP-1’s role in phase shifting, it does not preclude the possibility that tPA and LRP-1 interact when tPA is present, particularly given the many ways tPA and LRP-1 can interact. Given the diurnal variations we see in αLRP-1, the increase in αLRP-1 at night could influence tPA activity in a variety of ways (Figure 4.1). First, it could allow LRP-1 to clear tPA from the extracellular space, either before or after it is complexed with an inhibitor. LRP-1 recycling of tPA could control amounts of tPA in the extracellular space in the SCN, thus controlling its proteolytic activity. Alternatively, LRP-1 may enhance tPA’s proteolytic activity by acting as a co-receptor. One study found that LRP-1 acts as a co-receptor to enhance tPA cleavage of platelet derived growth factor-CC (PDGF-CC) in primary cultured cortical microglia (Su et al., 2017). LRP-1 could use similar processes to regulate uPA or MMP activity in the SCN, as it binds and endocytoses them as well. Regardless of the specifics, LRP-1 dependent regulation of extracellular proteolytic activity could align nicely with our studies on LRP-1 and the plasminogen activators in the SCN.

4.2.5 LRP-1 influences expression and function of ECM-interacting proteins

A second way LRP-1 influences ECM composition is by regulating ECM-interacting proteins. Cell-associated proteins that can be regulated by LRP-1 include integrins and uPAR, which may have both independent and overlapping roles. Interactions with these two molecules are points of overlap between LRP-1 and uPA that will also be highlighted here.

uPAR

uPAR is a GPI-anchored membrane receptor that is important for many uPA functions. uPA binding to uPAR allows plasmin to cleave uPA into its active form, thus increasing uPA proteolytic activity (Lijnen et al., 1987a; Lijnen et al., 1987b). Additionally, uPAR can mediate signaling events through co-receptors (Lino et al., 2014). LRP-1 and β1-integrin are two co-receptors that enable uPAR signaling events in neuronal systems, particularly in promoting axonal recovery following a CNS injury (Merino et al., 2017a). LRP-1’s interactions with uPAR are complex. In some contexts, LRP-1 modulates uPAR surface localization by endocytosing uPA-serpin complexes that are simultaneously bound to uPAR and LRP-1 (Conese et al., 1995). This can serve to control uPA and uPAR
During the day, when lower levels of αLRP-1 are detected, LRP-1 may serve to endocytose extracellular tPA. At night, when levels of αLRP-1 are high, it may also endocytose tPA, but there could be increased recycling back to cell surface. tPA levels are higher at night, and this could stimulate LRP-1 endocytic activity, shedding, or signaling events. Additionally, LRP-1 may act as a co-receptor increasing tPA proteolytic activity at night.
activity, as discussed above. LRP-1 can also mediate cell signaling through uPAR. In other contexts uPA binding to uPAR doesn’t result in LRP-1 mediated endocytosis of the complex, but rather initiates LRP-1 dependent recruitment of β1-integrin to the neuronal membrane (Merino et al., 2017a). The uPA-uPAR-LRP-1-integrin complex then mediates Rac1 activation, which can influence axonal regeneration (Merino et al., 2017b). Separately, binding of uPA to uPAR increases their affinity of uPAR for vitronectin and integrins, which promotes cell adhesion (Kanse et al., 1996; Etique et al., 2013).

Interactions via uPAR could serve as a link between uPA and LRP-1 in the SCN. First, involvement of uPAR may underlie uPAs compensatory action on circadian clock phase shifting, which remains elusive. We have preliminary data suggesting uPAR is expressed in the SCN, and that its expression levels do not exhibit circadian rhythms. However, a functional assessment of uPAR in the SCN has yet to be completed. Future experiments could address uPAR involvement in circadian clock phase shifting, investigate interactions between uPAR and LRP-1 proteins, and determine if inhibiting LRP-1 influences uPAR expression in the SCN.

**Integrins**

LRP-1 and uPA function are also linked through interactions with integrins. Not only do they work in concert with uPAR to influence integrin trafficking, as discussed above, but they also each influence integrin function in other ways. Integrins are heterodimeric transmembrane receptors that mediate cell-cell and cell-ECM adhesions. Integrins are expressed in mature synapses, where they coordinate synapse structure and function in response to changes in the extracellular environment. Integrins regulate synaptic transmission by affecting synaptic strength and neuronal excitability (Park & Goda, 2016). For example, they can control the number and composition of AMPA receptors (Pozo et al., 2012), and can enhance NMDAR activity by regulating phosphorylation of GluN2A and GluN2B NMDAR subunits (Chavis & Westbrook, 2001; Shi & Ethell, 2006). Changes in integrin binding also leads to changes in dendritic spine shape (Park & Goda, 2016). Additionally, they are necessary for MMP9 to increase lateral diffusion of GluN1 subunits between synaptic and extrasynaptic sites, which influences NMDAR responses to glutamate (Michaluk et al., 2009). Collectively, integrins seem to be a crucial member of the extracellular milieu that influences neuronal plasticity.
uPAR interacts with various integrin subunits, including β1, β3, and β6, and these interactions can mediate some of uPARs neuronal functions (Eden et al., 2011). β1 integrin is a receptor for fibronectin, which is required for uPA/uPAR functions such as promoting axonal regeneration (Diaz et al., 2017). uPA induces recruitment of β1 integrin to the plasma membrane in cerebral cortical neurons, and β1 integrin neutralizing antibodies block effects of uPA-uPAR binding on axonal repair (Merino et al., 2017a). So, interactions between β1 integrin and fibronectin mediate uPA-induced neurorepair.

LRP-1 also associates with integrins, and this interaction can modify integrin activation, trafficking, degradation, and downstream signaling (Wujak et al., 2017). LRP-1 mediates integrin activity by mediating their internalization or influencing their maturation and localization to the cell surface (Lillis et al., 2008; Wujak et al., 2017). In some cases, this involves interactions with uPAR (Czekay & Loskutoff, 2009). In other cases, it is a direct association, such as when LRP-1 associates with β2-integrins on leukocytes; this interaction is thought to regulate integrin recycling during macrophage migration (Cao et al., 2006). LRP-1 can also play a role in delivery of integrins to the cell surface (Theret et al., 2017). Loss of LRP-1 correlates with reduced cell-surface expression of β1-integrin, but not total β1 integrin (Salicioni et al., 2004; Spijkers et al., 2005; Cao et al., 2006). Interestingly, RAP does not affect integrin maturation, suggesting that LRP-1’s regulation of integrin maturation does not require ligand binding or endocytosis (Salicioni et al., 2004). LRP-1 could associate with chaperones or adaptor proteins to mediate this effect, because LRP-1 is not co-immunoprecipitated with β1 integrin. Chaperones or adaptor proteins such as hsp90, Fe65, or ICAP-1 might act as a bridge between LRP-1 and integrin (Salicioni et al., 2004), and LRP-1’s effects sometimes depend on other molecules such as thrombospondin, tPA. Integrins have not yet been assessed in the SCN. However, given their ability to modulate neuroplasticity, the involvement of other cell adhesion molecules (CAMs) in the SCN, and their close associations with proteins in this research, an investigation of integrins in the SCN seems warranted.

4.3 To shift or not to shift – decision making in the SCN

One key question remaining regarding SCN timekeeping is how it generates such dramatically different responses to stimuli over the course of the day. The same stimulus (light or glutamate) induces phase advances and delays when applied at night, but not
during the day, indicating that there are 24 hr cycles in neuronal responsiveness in the SCN. The current models explaining this plasticity focus on several proteins, including BDNF, TrkB, NMDARs, and various intracellular effectors. However, many modulators of neuroplasticity identified in other regions remain uninvestigated in the SCN. The ECM-associated processes and astroglial rhythms discussed above likely contribute to the daily plasticity in the SCN. Additionally, our finding that inhibiting LRP-1 prevents glutamate-induced phase delays suggests that the ECM may contribute to this decision making process in the clock. Because of LRP-1’s complexity, it implicates many molecules as potential regulators of this function. Above, I discussed ECM associations as a potential mechanisms underlying LRP-1's role in phase shifting, and here I will focus on receptor interactions that could enable these phase shifting decisions. In particular, LRP-1 may act by modulating NMDAR calcium signaling in the SCN.

4.3.1 LRP-1 and NMDAR

NMDARs modulate neuroplasticity throughout the CNS, and in the SCN clock phase shifting relies heavily on NMDAR signaling. NMDAR-based calcium-influx is required for glutamate-induced phase shifting, as inhibiting NMDARs prevents these phase shifts and NMDA administration induces night-time phase shifts (Colwell, 2001). While there are data supporting rhythms in NMDAR expression patterns and phosphorylation in the SCN (Bendova et al., 2012), many questions remain unanswered regarding how NMDARs are regulated in the SCN, and thus how they contribute to daily iterative changes in neuronal responsiveness.

We have found that inhibiting LRP-1 prevents glutamate induced phase shifts, which means that we are preventing a process that depends on NMDAR signaling. Inhibiting LRP-1 attenuates NMDAR calcium influx in other systems (Mantuano et al., 2013). Thus, the mechanism through which LRP-1 is acting may involve changes in NMDAR responses, which could be achieved through several mechanisms. A first set of possibilities relates to a direct influence of LRP-1 on NMDAR localization (Figure 4.2). LRP-1 can physically connect to NMDAR via PSD95 (May et al., 2004). LRP-1 can regulate the surface distribution and internalization of NR2B-containing NMDAR receptors, which may be the source of its permissive role in phase shifting (Maier et al., 2013). One possible model is that LRP-1 increases the amount of NR2B-containing
Figure 4-2. LRP-1 NMDAR interactions

Models demonstrating ways that LRP-1 could influence NMDAR signaling. Synaptic localization: During the day, LRP-1 may mediate internalization of NMDARs or lateral diffusion away from the synapse, thus preventing phase shifts to glutamate. LRP-1 may recruit NMDARs to the synapse during the night, enabling shifts. Co-receptor recruitment: During the day, LRP-1, NMDAR, and TrkB function independently. At night, increased tPA stimulates LRP-1 recruitment of NMDAR via interactions with PSD-95, and transactivates TrkB receptors, enabling phase shifting responses to glutamate.
NMDARs on the cell surface during the night, placing them in a prime location to be activated by glutamate and thus allow phase shifts to occur. During the daytime LRP-1 may mediate the internalization of NMDARs, thus attenuating glutamate-induced responses. Our finding that inhibiting LRP-1 with RAP changes NR2B subunit phosphorylation patterns supports the idea that LRP-1 influences NMDAR surface localization. However, our data are slightly counter-intuitive. We find a decrease in phosphorylation on S1480 when we inhibited LRP-1 with RAP, which has been found to correlate with increased rather than decreased NMDAR cell surface localization. The original study assessing LRP-1 influence on NMDAR localization also found patterns of NMDAR phosphorylation that did not align with their receptor localization data demonstrating a reduction in NMDARs on the cell surface (Maier et al., 2013). Regulation of NMDAR localization is complex, and it is possible that LRP-1 circumvents classical surface localization mechanisms by physically pulling NMDARs into the cell during their endocytic activities. A second possibility relates to the finding that LRP-1 can localize both in lipid rafts and in clathrin coated pits, and can move laterally in and out of the synaptic regions (Laudati et al., 2016). Thus, instead of internalizing NMDARs, LRP-1 could also change their synaptic vs. extrasynaptic localization, and these changes may regulate glutamate phase shifting responses across the day. Synaptic vs extrasynaptic localization is an additional mechanism that influences glutamate signaling responses (Sanz-Clemente et al., 2013). Directly assessing cell surface dynamics of NMDARs and LRP-1 over the course of the day and/or in response to RAP treatment could provide some insight into these scenarios in the SCN.

Finally, although it has not been investigated with respect to LRP-1 activity, another key regulator of NMDAR signaling properties is NMDAR subunit composition. NMDAR subunit composition varies throughout the CNS and changes depending on neuronal activity. NMDARs consist of GluN1 subunits paired with one of four GluN2 subunits (GluN2A-D). The four GluN2 subunits are largely responsible for functional heterogeneity. GluN2A and GluN2B are thought to have central roles in synaptic plasticity (Paoletti et al., 2013). NMDAR subunit composition also varies according to cellular localization. Generally, synaptic NMDARs contain GluN1/GluN2A, and heterotrimeric GluN1/GluN2A/GluN2B receptors, while extrasynaptic NMDARs contain a higher proportion of GluN2B subunits, although this is a drastic oversimplification. NMDARs are
mobile, and lateral diffusion can contribute to changes in NMDAR activities (Paoletti et al., 2013). NMDAR subunit composition is highly plastic, and changes in subunit composition is an additional mechanism fine-tuning NMDAR responses (Paoletti et al., 2013). Individual NMDAR subunits exhibit rhythmic patterns of expression in the SCN. In particular, total NMDAR and NR2A subunit expression is higher at night, and in general NMDAR activity is higher at night than in the day in the SCN (Bendova et al., 2009). Given the ability of LRP-1 to affect so many other NMDAR functions, it is possible that it contributes to the recruitment of specific NMDAR subunits – a concept that has yet to be explored.

4.3.2 LRP-1 – NMDAR – Trk receptor complexes and their relevance to clock phase shifts

In addition to influencing NMDAR localization, LRP-1 acts in conjunction with NMDARs and Trk receptors as co-receptors to induce signaling events in response to extracellular ligands (Mantuano et al., 2013). For example, NMDAR can function as an LRP-1 coreceptor to promote Schwann cell survival and migration (Mantuano et al., 2015). LRP-1, NMDAR, and Trk receptors assemble to form a unique co-receptor system that integrates signaling events in response to LRP-1 ligands (Mantuano et al., 2013). LRP-1 cell signaling is ligand dependent, and this may be in part because different ligands induce different receptor co-recruitment (Mantuano et al., 2013).

Another important LRP-1:Trk ligand is α2-macroglobulin. The ability of α2-macroglobulin to promote neurite outgrowth on PC12 cells, N2a cells, and cerebellar granule neurons requires both LRP-1 binding and Trk receptor transactivation via SFKs (Shi et al., 2009). α2-macroglobulin binding to LRP-1 induces Trk phosphorylation in an SFK dependent manner. SFK antagonism or Trk receptor inhibition prevents the responses mediated by α2-macroglobulin and tPA, which includes ERK1/2 activation and neurite outgrowth. So, α2-macroglobulin stimulated LRP-1-dependend Trk transactivation may be a distinct pathway influencing cell signaling without NMDARs (Rebeck, 2009). In PC12 and N2a neuron-like cell culture tPA induces ERK1/2 activation in two phases, one that is LRP-1 dependent and rapid, and a slower one that is independent of LRP-1 (Mantuano et al., 2013). The LRP-1 dependent phase involves both NMDAR and Trk receptors, which function as a single signaling system (Mantuano et al., 2013). Inhibiting
either NMDAR with MK801 prevents tERK1/2 activation, and it prevents tPA or α2-macroglobulin induced phosphorylation of Trk receptors (Mantuano et al., 2013). So the data support a model where some LRP-1 ligands (but not all), recruit NMDARs as a coreceptor to stimulate transactivation of Trk receptors, with the conclusion being that all three work in conjunction to mediate ERK1/2 activation (Mantuano et al., 2013). It is also interesting to note that PSD-95 is recruited to LRP-1 following tPA or α2-macroglobulin treatment. While this was demonstrated with TrkA receptors in neuron-like cells, it is plausible that a similar receptor platform involving TrkB receptors mediates signaling in the SCN. Each of these proteins is implicated independently in gating phase shifting: tPA levels increase at night and this is necessary for glutamate-induced phase shifts in vitro, NMDARs are the primary receptor mediating photic/glutamate phase shifts, TrkB receptors are necessary for glutamate phase shifts, and here we have demonstrated that LRP-1 is also required for phase shifting. A possible model is that high night-time tPA expression allows it to bind to LRP-1, mediating signaling events that require both NMDAR and TrkB receptors, that ultimately lead to downstream signaling events (Figure 4.2). The recruitment of this complex could also serve to increase neuronal responses to glutamate. As with the other possibilities described, this model could overlap with additional models (Figure 4.3).

**4.3.3 LRP-1 and AMPA**

Finally, it is worth mentioning that LRP-1 can also interact with AMPA receptors. AMPARs are also important for LTP and LTD and are regulated largely through phosphorylation and de-phosphorylation of their c-terminal domain (Lee et al., 2000). AMPA receptors traffic rapidly, moving through endocytic pathways, sorting to degradation pathways or being recycled back to the plasma membrane (Huganir & Nicoll, 2013), where they influence dendrite and spine motility and contribute to synaptic plasticity. AMPA receptors participate in photic phase shifts by acting upstream of NMDAR, probably by removing a magnesium block (Mintz et al., 1999; Mizoro et al., 2010). Conditional neuronal knockout of the Lrp1 gene decreases level of GluA1 mRNA and protein in the brain (Liu et al., 2010). LRP-1 can also influence the cellular distribution, turnover and phosphorylation of GluA1, and this influences calcium influx, neurite outgrowth, and filipodia formation in neurons (Gan et al., 2014). GluA1, LRP-1 and PSD95 form
LRP-1, tPA, and uPA interact in a variety of ways to mediate glutamate-induced phase shifting in the mammalian SCN. Known interactions include tPA or uPA plasmin-dependent generation of mBDNF, activating TrkB receptors, and enabling NMDAR-dependent glutamate-induced phase shifts. LRP-1 is also required for phase shifting in vitro, but the mechanism is unclear. Potential interactions relevant to SCN clock function include associations with NMDARs via PSD95 and transactivation of Trk receptors. Ligand binding to LRP-1 may result in a variety of consequences, including ligand endocytosis, recycling, degradation, and/or activation of intracellular signaling. Shed LRP-1 in the extracellular space may also impact LRP-1 ligand function. Interactions with uPAR or integrins could also regulate phase shifts, and their role could involve interactions with the ECM. Much more work is necessary to fully understand how these proteins act in concert to coordinate phase shifting responses in the SCN.
complexes, similar to those seen with NMDARs, and these could influence AMPA receptor recycling (Gan et al., 2014). Whether or not LRP-1 is influencing AMPARs in the SCN is another question that remains to be explored.

### 4.3.4 Global considerations for LRP-1

Although our study of LRP-1 focuses on SCN timekeeping mechanisms, a more global role for LRP-1 in daily rhythmicity throughout the body should not be ignored. LRP-1 responses are largely context dependent, and circadian rhythms in cellular signaling seem to be the norm, not an exception. With that in mind, understanding how LRP-1 functions across the day may be important to understand its physiological influence. Additionally, our data indicate LRP-1 does not simply respond passively to extracellular changes, but serves to regulate time-keeping decisions. If we are to extrapolate this finding to the remainder of the body, it will be important to understand how circadian timing influences LRP-1 effects, and LRP-1 influences the timing of physiology. With that in mind, there are several tantalizing connections for which LRP-1 and an understanding of LRP-1 in clocks could have translational benefits.

### 4.3.5 Metabolic syndrome

First, disruptions in both LRP-1 and circadian rhythms are associated with development of metabolic syndrome, which is a series of physiological, metabolic, and biochemical risk factors for type 2 diabetes and cardiovascular disease (Au et al., 2017). LRP-1 is involved in insulin signaling and glucose homeostasis, both of which are massively disrupted in metabolic syndrome and associated pathologies (Au et al., 2017). Shift work disorder (a case of chronic circadian disruption) is also associated with an increased prevalence of metabolic syndrome (Tarquini & Mazzoccoli, 2017). Glucose homeostasis also contributes to SCN clock function, and in turn the clock regulates daily timing of glucose metabolism (Ruiter et al., 2006; Dibner & Schibler, 2015). Much more work needs to be done to fully connect the pieces of this enormous puzzle, but our data demonstrating a role for LRP-1 in circadian rhythms suggest that it could be a prime candidate linking the processes.
4.3.6 Alzheimer’s disease

LRP-1 and circadian rhythms also overlap in the context of Alzheimer’s disease development and progression. LRP-1 regulates the metabolism of amyloid-β, and preclinical studies suggest that LRP-1 plays a role in regulating apolipoprotein-E (APO-E) pathogenesis, though the precise roles of LRP-1 remain elusive in this context as well (Shinohara et al., 2017). Circadian disruptions, notably changes in the sleep-wake cycle, are also associated with neurodegenerative disorders such as Alzheimer’s disease; though the jury is still out regarding which component is the causative agent (Musiek & Holtzman, 2016). Given that LRP-1 is being proposed as a therapeutic target for AD, and that the roles of LRP-1 are context dependent (Shinohara et al., 2017), the circadian contributions to LRP-1 function, both in the brain and throughout the periphery may be important to untangle for optimal therapeutic benefit. With the intriguing proposal that Alzheimer’s disease can be characterized as type 3 diabetes because of insulin dysregulation in the brain, the associations between LRP-1, circadian rhythms metabolic disorders and dementia increase (de la Monte & Wands, 2008).

4.4 Final conclusions

In conclusion, here we have presented two independent studies, one finding that uPA can compensate to allow phase shifting in tPA−/− mice, and the other showing that LRP-1 is necessary for glutamate induced phase shifts of SCN neuronal activity rhythms in vitro. While the plasminogen activators and LRP-1 overlap in some functions, they also have distinct effects on neuronal systems, and it will be important to untangle the mechanisms underlying the roles of each in the SCN. While uPA expression is low in the brain, the finding that uPA is required for a normal neuronal response in tPA−/− mice suggests that a closer look at uPA’s physiological role, in addition to its pathological roles, may be warranted.

On a different note, our finding that LRP-1 is involved in circadian clock phase shifting the first indication of a protein with its capabilities involved in timekeeping mechanisms. LRP-1 (and other LDL receptors) are unique in their endocytic and signaling capabilities. LRP-1 binds a large and diverse range of ligands, and mediates a complex array of events following ligand binding, including endocytosis, signal transduction, and co-receptor recruitment. LRP-1 functions are context dependent, and in the SCN context
changes dramatically over the course of 24 hours. LRP-1 could be simply responding to the changing extracellular environment of the SCN, nonselectively binding ligands based on what is most available in the ECM. However, such a passive role doesn’t explain why blocking ligand binding prevents phase shifts. This result suggests that somehow LRP-1 is communicating information to make a decision on whether or not to shift. Understanding what ligands and what intracellular effectors are involved will be important to fully understand LRP-1’s role in the clock, where LRP-1 may serve as both a surveyor and regulator of extracellular space in the SCN.
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VITA

Joanna Marie Cooper was born in Fayetteville, North Carolina to Margaret and Danny, as the younger sister to Melissa, and eventually the older sister to Matthew. She grew up in a military family, and lived in Bad Tölz, Germany and Massachusetts before returning to Harnett County, North Carolina. She graduated from Western Harnett High School in 2005, and started her undergraduate studies at the University of Alabama in Huntsville, where she completed 1.5 years of undergraduate work before transferring to the University of North Carolina at Pembroke (UNCP). Her path to become a scientist was circuitous: she entered the nursing program at UNCP in 2009, and completed two semesters of the program before she realized her passion was in understanding the processes underlying disease. Joanna left the nursing program and changed her major to biology. During her time as an undergraduate, she was inspired to pursue neuroscience research through two experiences. First, as a part of the Research Initiative for Scientific Enhancement (RISE) program at UNCP she studied under Dr. Ben A. Bahr researching lysosomal modulation as a potential therapeutic for Alzheimer’s disease and lysosomal storage disorders. Second, she participated in the University of Pittsburgh Center for Neuroscience Summer Internship Program in Pittsburgh, Pennsylvania, where under the supervision of Dr. Timothy A. Greenamyre and Dr. Laurie H. Andolina she researched DNA repair processes in Parkinson’s disease. Joanna earned a BS in Biology from UNCP in 2011, and entered the BCMB program at the University of Tennessee, Knoxville. As a graduate student, Joanna continued to pursue her passion for understanding the brain in Dr. Rebecca Prosser’s lab, where she learned about circadian rhythms, embarked on a study of the intriguingly complex LRP-1 in the suprachiasmatic nucleus, and uncovered a role for uPA in the clock along the way. Joanna accepted a postdoctoral position with Dr. Dudley Strickland at the University of Maryland School of Medicine in Baltimore, MD.